Quantitative detection of *Salmonella enterica* and the specific interaction with *Lactuca sativa*

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2007

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Dit onderzoek is uitgevoerd binnen de C.T. de Wit Onderzoekschool 'Productie Ecologie en Beheer van Natuurlijke Hulpbronnen'

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. M. J. Kropff,
in het openbaar te verdedigen
op woensdag 20 juni 2007
des namiddags te 13.30 uur in de Aula

Michel M. Klerks (2007)

Quantitative detection of *Salmonella enterica* and the specific interaction with *Lactuca sativa.*

Doctoral thesis - Biological Farming Systems Group - Wageningen University - The Netherlands.

Subject headings*: Salmonella enterica*, *Escherichia coli*, detection, lettuce, plant response, pathogenicity, route of infection

ISBN: 978-90-8504-674-5

Contents

Abstract

In recent years the number of enteric disease outbreaks has increased, partly due to an increase in human pathogenic bacteria associated with fresh produce. The use of manure for production of vegetables, e.g. lettuce, contributes significantly to the risk of contamination of fresh produce. Enteric pathogens like *Salmonella* associated with manure can come in close contact with plants like lettuce, and a better understanding of the interaction between lettuce and *Salmonella enterica* serovars during lettuce cultivation is necessary to be able to take preventive actions to reduce the risk for human health.

This thesis describes a comparison of different molecular methods to detect *Salmonella enterica* (*invA*-gene) or *Escherichia coli* O157:H7 (*stx*-1, *stx*-2 and *eae*-gene) with respect to sensitivity, precision and accuracy. The detection and quantification methods were improved by the addition of a general internal amplification control. The methods enable a reduction in assay time to two days to test food samples, compared to five days required for the standardized procedures. To improve detection of *S. enterica* serovars from environmental substrates, five DNA extraction methods were evaluated with respect to DNA extraction efficiency from soil, manure and compost. Inclusion of an internal procedural control permitted a more accurate quantification of *S. enterica* after DNA extraction and amplification and reduced the possibility of false-negatives.

This thesis also describes the physiological and molecular interaction between *S. enterica* serovars and lettuce. Investigation of the localization of *S.* Dublin on/in lettuce plants revealed the presence of significant populations on the surface and inside the plants. Next to this physiological response, there were clear differential gene expression profiles between non-colonized and colonized lettuce plants based on transcriptome analysis by cDNA-AFLP. Functional grouping of the expressed genes indicated a correlation between colonization of the plants and an increase in expressed pathogenicity-related genes. Moreover, a differential interaction of *S*. Typhimurium, *S.* Enteritidis, *S*. Dublin, *S.* Newport and *S.* Montevideo with lettuce cultivars Cancan, Nelly and Tamburo was found, in terms of prevalence and degree of endophytic colonization of lettuce by the *S. enterica* serovars. Besides a significant interaction, significant differences among serovars, but not among lettuce cultivars, were obtained when lettuce was grown under axenic conditions. When grown on soil, each evaluated *Salmonella* serovar was able to colonize lettuce epiphytically, but to a lower extent than on axenically grown plants. Only *S.* Dublin was able to colonize the plants endophytically when these were grown on contaminated soil. In addition, the active movement of *S. enterica* serovars towards lettuce roots is presented. Micro-array analyses with DNA extracted from a broth culture of *S.* Typhimurium with or without exudates identified

genes of *S. enterica* serovars that were induced by root exudates. These genes imply a relation with a sugar-like carbon source. From this study different plant and microbial factors that influence the colonization efficiency of *S. enterica* serovars have been identified. The serovar and cultivar, but indirectly also the rhizosphere and the endophytic microflora of lettuce were most influential with respect to the risk of colonization and thus the risk for human health.

Chapter 1

General Introduction

General introduction

Produce-associated outbreaks caused by Salmonella enterica *or* E. coli *O157:H7 contamination*

In recent decades an increase in disease outbreaks has been recognized to be caused by human pathogenic bacteria in association with fresh produce (Sivapalasingam *et al.*, 2004; Lyytikianen *et al.*, 2004; Viswanathan and Kaur, 2001). In the period from 1973 through 1997, at least 190 reported outbreaks in the US were associated with the consumption of fresh produce, causing 16058 illnesses, 598 hospitalizations and eight deaths (Sivapalasingam *et al.*, 2004). From this review it was stated that in the 90s at least 6% of all foodborne outbreaks were related to contaminated fresh produce, compared to 0.7% in the 70s. Among the 103 outbreaks with known pathogens, 62 (60%) were caused by bacterial pathogens of which 30 (48%) were caused by *Salmonella enterica* serovars and 13 (20%) by *Escherichia coli* O157:H7. Of all produce-associated outbreaks the most commonly found pathogen was *Salmonella.* In this respect, it should be mentioned that of all incidences of Salmonellosis, up to 91.6% is not reported because patients frequently do not visit a physician (Figure 1). This means that the actual number of incidences is much higher than those reported.

Several Salmonellosis outbreaks have occurred that were associated with the consumption of fresh produce (International Society for Infectious Diseases, ISID). For example, in 2004 two outbreaks were reported from Norway and England, of which 13 and 368 cases were reported, the latter outbreak leading to 33 hospitalizations. Another Salmonellosis outbreak in 2006 was reported from Sweden (100 cases) and was attributed to consumption of mung bean sprouts. Most recently (2006), an outbreak of Salmonellosis was reported from the US (106 cases, of which 40 were hospitalized) that was linked to consumption of tomatoes. A recent (2006) multi-state outbreak of *E. coli* O157:H7 obtained much attention worldwide since many people became seriously ill from the consumption of contaminated spinach. At least 187 cases were reported related to this outbreak, of which 97 hospitalizations, 20 cases of hemolytic uremic syndrome, and 1 death.

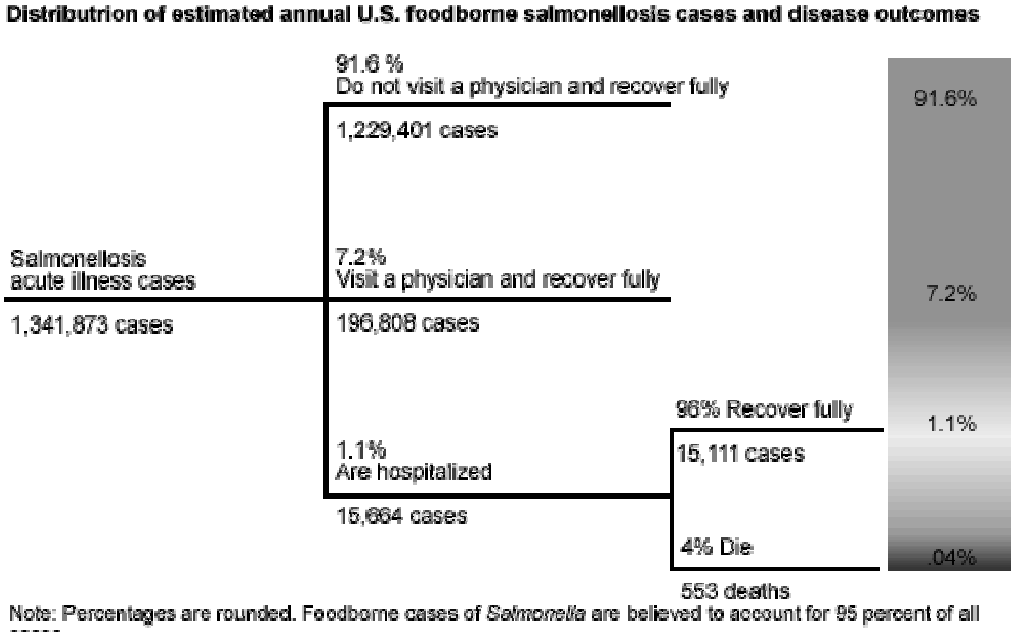

Figure 1. *Source: USDA Research service, Foodborne illness Cost Calculator 2003; http://www.ers.usda.gov/Data/FoodBorneIllness*

Different types of produce have been implicated in foodborne outbreaks in the US of which lettuce, melon and sprouts were found most often as the vehicle for disease (Sivapalasingam *et al.*, 2004). Specifically for *Salmonella*, produce-associated outbreaks were most often caused by contaminated salad, sprouts and melons. As for *E. coli* O157:H7 the outbreaks were most often caused by contaminated lettuce and apple cider. Most outbreaks related to consumption of fresh vegetables have been reported for *S. enterica* and to a lesser extent *E. coli* O157:H7 (Table 1). Therefore, this thesis is mainly focused on *S. enterica*. As several foodborne outbreaks were associated with consumption of lettuce contaminated with *S. enterica*, lettuce was selected as model plant for this thesis.

For greenhouse grown produce, enteric pathogens are suggested to be introduced as a result of bad hygiene (Beuchat and Ryu, 1997; Anonymous, 1999). In the field however, contamination of vegetable crops may also occur via soil amended with manure from agricultural animals which are known reservoirs for Salmonellae (Natvig *et al.*, 2002; Viswanatan and Kaur, 2001). Both manure and irrigation water are thought to contribute significantly to the spread of human pathogens to fields and the crops growing there (Islam *et al.*, 2004; Solomon *et al.*, 2002). Manure is known to harbor high numbers of human pathogenic bacteria like *S. enterica* and *E.coli* O157, which can remain viable for extensive periods of time, even up to one year (Baloda *et al.*, 2001; Kudva *et al.*, 1998; Wang *et al.*,

1996). Even when applying artificially infested manure to soil, the number of enteric bacteria was reduced only 1 order of magnitude after a period of three months (Franz *et al.*, 2005). Thus, contamination of plants with human pathogenic bacteria from manure may occur, for example during rainfall or irrigation due to splashing of soil and bacteria onto the plants (Natvig *et al.*, 2002).

Table 1. *Type of produce items implicated in foodborne outbreaks by pathogens in the US, 1973 through 1997.*

Salmonella enterica		***E. coli* O157:H7**		**Other bacteria**	
Produce item	Number of outbreaks	Produce item	Number of outbreaks	Produce item	Number of outbreaks
Salad	7	Lettuce	5	Carrot	1
Seed sprouts	7	Apple cider	4	Pineapple	1
Melon	6	Salad	2	Salad	6
Apple or orange juice	3	Cantaloupe	1	Lettuce	2
Lettuce	3	Alfalfa sprout	1	Green onion	1
Tomato	1			Mixed vegetables	1
Lettuce and tomato	1			Melon	3
Precut celery	1			Fruit salad	1
Mixed fruit	1			Sprouts	1
				Bean sprouts	1
				Strawberry	1
Total	30		13		19

Detection methods

Due to the high prevalence of *Salmonella*- and *E.coli* O157:H7-associated foodborne outbreaks, there was a need for certified identification and detection procedures. In the late 1950s a standardized protocol was developed based on plating on selective medium. Although many optimizations have occurred, certification procedures to detect the presence of *S. enterica* or *E. coli* O157:H7 in food are still based on the standardized microbiological culturing methods (ISO 6579/2002 and ISO 16654/2001, respectively). A major advantage of this approach is that a-selective enrichment is required prior to selective analysis, herewith generating sufficient material for downstream characterization and identification. In addition, only viable bacterial cells are detected.

However, this methodology requires up to five days until results are obtained (Stewart *et al*.,

1998). With respect to food quality and safety issues, the assay time exceeds the shelf-life or "best-before" dates of vegetables consumed fresh. In order to reduce the time demand, different techniques have recently been applied to detect *S. enterica* or *E. coli* O157:H7 from food samples, like immunological assays (Acheson *et al*., 1994; Bolton *et al*., 2000) and molecular methods (Bej *et al*., 1994; Heller *et al*., 2003; De Medici *et al*., 2003). Especially real-time PCR methods such as 5' nuclease (Taqman) PCR (Holland *et al*., 1991) and PCR combined with molecular beacons (Tyagi *et al*., 1996), have shown promising results due to the rapid, sensitive and specific detection of pathogens (Higgins *et al*., 1998; Bassler *et al*., 1995). Such real-time PCR methods have also been reported for the detection of *S. enterica*, primarily based on the amplification of species-specific genes, or genes related to pathogenicity or virulence. For example, the detection of *S. enterica* was accomplished by real-time PCR methods directed to the *spaQ*-gene (Kurowski *et al*., 2002), the *invA*-gene (Hoorfar *et al*., 2000) and the *himA*-gene (Chen *et al*., 2000). Also for the detection of *E. coli* O157:H7 real-time Taqman PCR methods have been described, directed to the *rfbE*-gene (Fortin *et al*., 2001) and, in a simultaneous matter, the three Shiga toxin genes: Shiga toxin 1 (*stx1*-gene), Shiga toxin 2 (*stx2*-gene) and intimin (*eae*-gene) (Molecular beacon-based detection, Ibekwe *et al*., 2002; Taqman-based detection, Sharma *et al*., 1999).

Real-time PCR methods are often used for quantification of a target organism in a substrate sample. However, amplification efficiencies can be different from sample to sample due to the effects caused by inhibition of amplification, human failures or preparation errors. To eliminate part of these drawbacks, recently different approaches have been described that involve an internal amplification control in each real-time PCR (i.e. Hoorfar *et al*., 2000; Raggam *et al*., 2002; Savli *et al*., 2003; Vandesompele *et al*., 2002). Still, molecular methods are limited by the fact that these are dependent on the suitability of the extracted DNA for PCR (Wilson, 1997). Especially DNA extracted from soil, manure or compost, all three sources that introduce human pathogens in the production chain (Figure 2), can have co-extracted contaminants like humic and fulvic acids that are known to cause problems during PCR amplification (Al-soud and Rådström, 1998; Fortin *et al*., 2004). Other components different from humic and fulvic acids but commonly present in soil, have also been related to PCR inhibition (Watson *et al*., 2000). Moreover, the large variation in biochemical components between different substrates (Al-soud and Rådström, 1998; Wilson, 1997) usually leads to variable efficiencies of DNA extraction methods (Lloyd-Jones and Hunter, 2001; Theron and Cloete, 2000). Due to these deficiencies, accurate quantification of pathogens present in different environmental substrates has not yet been accomplished using molecular techniques such as PCR.

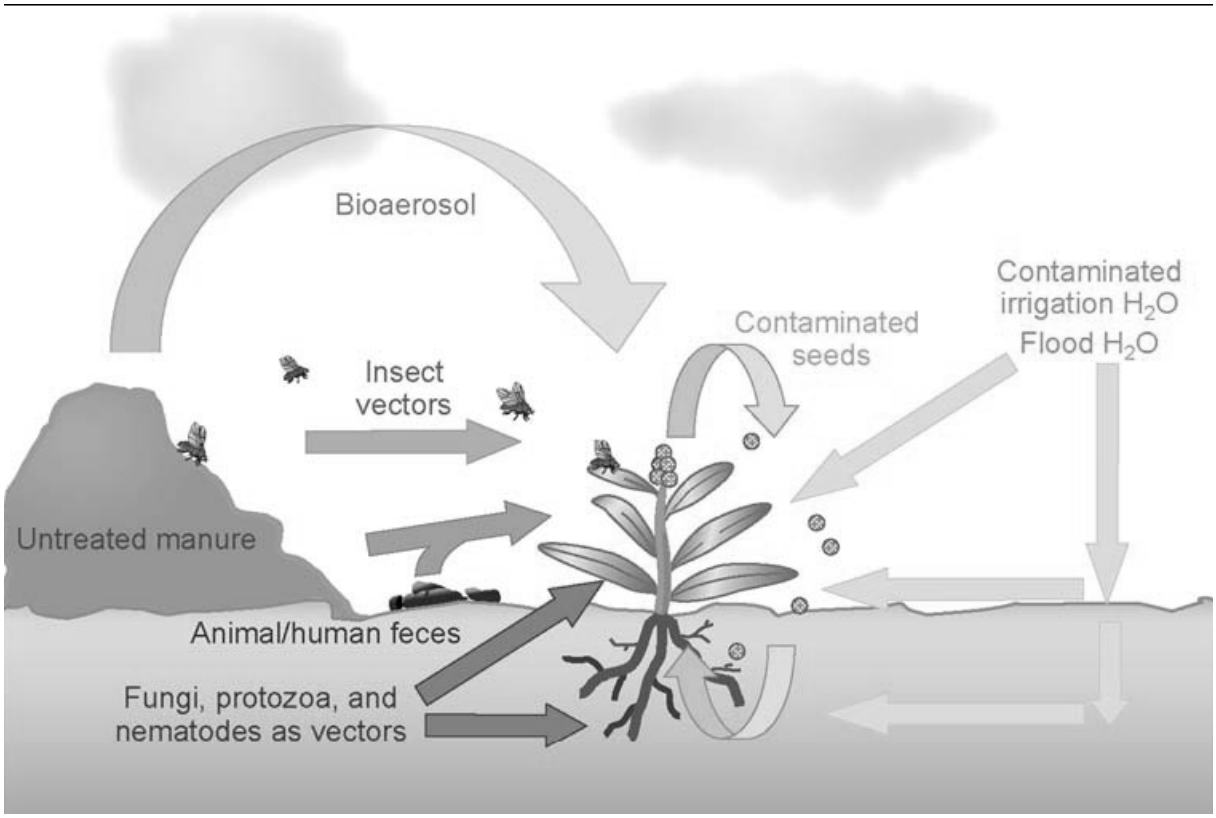

Figure 2. *"Schematic overview of factors that can contribute to the contamination of fruit and vegetables with human enteric pathogens in the field" (M. T. Brandl, 2006). The route of transmission of pathogens from manure to crops is mainly via soil or irrigation water, although also other factors can contribute to the contamination of field grown plants.*

To control the effects of inhibiting agents on PCR amplification efficiency, Taqman PCR was improved recently by introduction of a general internal amplification control to prevent the occurrence of false negative results (Hoorfar *et al.*, 2000; Raggam *et al.*, 2002). Although application of an amplification control provided progress in the analysis of extracted DNA from environmental substrates, no information was obtained with this control of the DNA extraction efficiency.

Moreover, the comparison between the DNA extraction efficiency of different DNA extraction methods has only been described to a minor extent. Zhou *et al*. (1996) investigated the DNA recovery from different soils, but only one DNA extraction method was used. Another paper described a comparison of three different DNA extraction methods, evaluating the DNA quality and quantity recovered from four soils with widely differing characteristics, but not from manure or compost (Lloyd-Jones and Hunter, 2001). The introduction of a control that gives an indication of both the extraction efficiency and the amplification efficiency would lead to more reliable quantification of the target organism from a specific substrate without the bias due to inhibiting agents or poor extraction efficiency.

Genus Salmonella

Salmonellae are gamma proteobacteria which were discovered for the first time in 1900 by the American bacteriologist Daniel Elmer Salmon, who isolated *Salmonella cholerae suis* from pigs. The bacteria are facultative anaerobic, Gram-negative, motile rods that cannot form spores. *Salmonella* spp. can infect a broad host spectrum, including mammals, birds and reptiles, and is associated with acute gastro-intestinal illnesses around the world (Baird-Parker, 1990). Each year Salmonellosis is responsible for 3.5 million cases in the US and Canada, which leads to economic losses up to 3.4 billion $ a year (Todd, 1989; ERS/USDA Foodborne illness Cost Calculator; *http://www.ers.usda.gov/Data/FoodBorneIllness/*). Salmonellosis is a zoönosal disease, meaning that pathogenic Salmonellae use animals as a reservoir and are transmitted to humans either directly, or via temporary vectors like water or produce.

Around 1930 the O- and H-antigens were used for the development of the so-called Kauffmann-White scheme, which enabled the differentiation between the increasing number of *Salmonella* species and subspecies (White, 1926; Kaufmann, 1978). The biochemical-based nomenclature (Figure 3) describes the genus *Salmonella* to consist of two species *Salmonella bongori* and *Salmonella enterica*. The latter is nowadays sub-divided in six subspecies (Anonymous, 2005; Tindall *et al.*, 2005), namely *S. enterica* ssp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*. The subspecies *enterica* comprises at least 2300 serovars, including for example the human pathogenic *S. enterica* ssp. *enterica* serovar Typhimurium or, in short, *S.* Typhimurium. To avoid misinterpretation, throughout this thesis the serovars of *Salmonella enterica*, ssp. *enterica* are designated by *'Salmonella* serovars'.

Almost all known human pathogenic *Salmonella* serovars are designated to the subspecies *enterica*. There is a remarkable variation in host range of the serovars of *S. enterica* ssp. *enterica*, from highly host-specific serovars to others that infect a broad spectrum of hosts. Although many virulence factors have already been identified, the differences in host-specificity and disease outcome between various serovars are not yet fully understood. It is generally assumed that horizontal DNA transfer, for example with pathogenicity islands, plays a major role for these differences in host specificity between the serovars (Rabsch *et al.*, 2002).

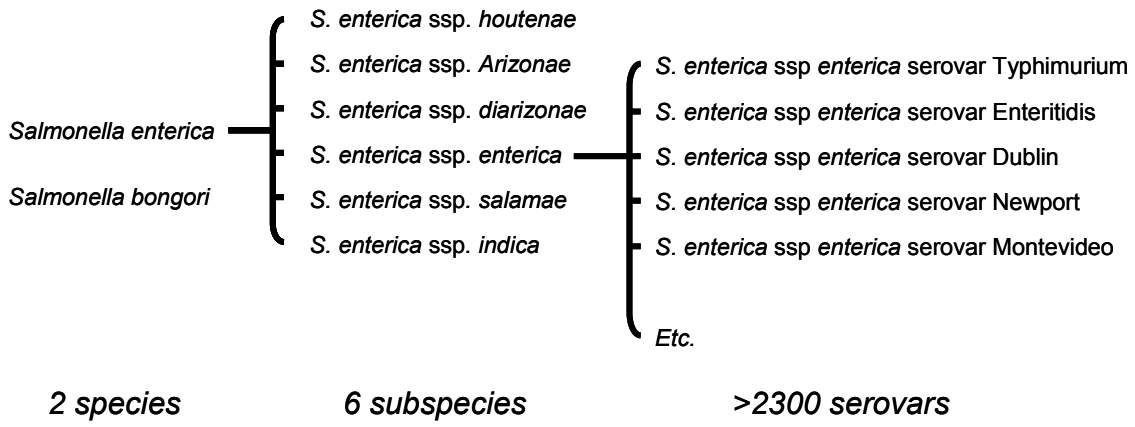

Figure 3. *Schematic representation of* Salmonella *nomenclature.*

Pathogenicity islands in bacterial pathogenesis

Gene loss and acquisition are the primary forces that allow bacteria to quickly genetically adapt to new environments and by which bacterial populations diverge and form separate, evolutionary distinct species (Groisman and Ochman, 1996). This event is essential for virulence of bacterial pathogens to animals and plants. In virulence studies of *E. coli* UPEC strains during the 1980s it was discovered that a single deletion event resulted in the loss of two linked virulence gene clusters, including an additional 30kb DNA segment (Hacker *et al.*, 1990; Blum *et al.*, 1994). This led to the definition of 'pathogenicity islands' (PAI). With these studies the deletion of a PAI resulted in a non-pathogenic phenotype of *E. coli*, from which it was suggested that such deletions were part of a genetic mechanism to modulate bacterial virulence.

In recent decades, many virulence factors have been identified and characterized that are located in PAI (an extended review was published by Schmidt and Hensel, 2004) and that are shared by plant and human pathogenic bacteria. Most of these pathogenicity factors interact with eukaryotic host cells, either by surface presentation or by being transported outside the bacterial cell. Secretion of proteins is a general requirement for bacteria, either pathogenic or non-pathogenic, to allow cell growth and metabolism (Thanassi and Hultgren, 2000).

Most virulence factors of *S. enterica* are coded by genes that are located within PAI on the bacterial chromosome, referred to as *Salmonella* pathogenicity islands (SPI) (Shea *et al.*, 1996). All genes that are required for invasion phenotype (needle structure, T3SS) are

clustered in a defined region of the chromosome, termed SPI-1 (Mills *et al.*, 1995). Besides SPI-1 also SPI-2 encodes for the T3SS to translocate virulence proteins into eukaryotic cells. Even though, the role in virulence is completely different between SPI-1 and SPI-2.

Invasion of eukaryotic cells that are non-phagocytic is dependent on the SPI-1 (Galan, 2001). The T3SS encoded by the SPI-1 has a needle-like structure (Kubori *et al.*, 1998) able to penetrate the host cell membrane. It can mediate the translocation of effector proteins into these host cells. A subset of these proteins is responsible for the re-organization of the actin cytoskeletal network of the eukaryotic cell by modifying the signal transduction pathways (Hardt *et al.*, 1998). The formation of actin filaments is induced at the site of translocation of the effector proteins that were responsible for the re-organization of the cytoskeleton. Upon formation of the actin filaments, the host cell membrane is ruffling to finally internalize the bacteria in a process called macropinocytosis. The regulation of the SPI-1 is not yet fully understood, although it has been recognized that the invasion genes that are located on SPI-1 are expressed dependent on the environment of the bacteria. Conditions like low oxygen level, high osmolarity or low pH result in expression of these genes following production of T3SS and secretory proteins and herewith leading to induced invasiveness.

The SPI-2 is essential for the ability to cause systemic infections and proliferation in animal organs (Hensel *et al.*, 1995). This event is linked to the possibility of *S. enterica* to survive in phagocytic cells and to replicate inside *Salmonella*-containing vesicles (SCV). The T3SS encoded by the SPI-2 is required for protection against host cell effector functions of the innate immunity while situated inside the SCV. It prevents the co-localization of the phagocyte oxidase (Vasquez-Torres *et al.*, 2000) and the inducible nitric oxide synthase (Chakravortty *et al.*, 2002) with the SCV. As a consequence, the intracellular *Salmonella* cells are protected from reactive oxygen intermediates, reactive nitrogen intermediates and the concurrent antimicrobial activity of peroxynitrite. The SPI-2 also contains genes that encode proteins for metabolic functions, like tetrathionate reductase (Hensel *et al.*, 1999). The availability of this enzyme allow *Salmonella* serovars to use tetrathionate as electron acceptor during anaerobic respiration. This might enable *Salmonella* cells to colonize anaerobic habitats.
Also another SPI is present in the *Salmonella* genome, designated SPI-3 (Blanc-Potard *et al.*, 1999). The SPI-3 encodes virulence factors that are mainly involved in a magnesium transport system, which is of importance for intracellular replication (Blanc-Potard *et al.*, 1999; Groisman and Ochman, 1996). Due to the low nutrient availability inside the SCV, a large set of metabolic pathways and transport systems is required for adaptation to this environment.

Salmonella serovars contain even more SPI's, from SPI-4 up to SPI-10. The role of these SPIs in *Salmonella* virulence has not yet been studied in full detail. However, they encode putative virulence factors which show sequence similarities to characterized virulence genes, effector protein genes or other genes related to virulence. To what extent these SPI's contribute to virulence in mammals, or non-hosts like plants, has yet to be determined.

To enable transport of intracellular molecules across the bacterial cell membrane into the extra-cellular environment, at least five different secretion systems are present in the membrane of the *Salmonella* cells. Due to the presence of an outer membrane in Gram negative bacteria these general secretion systems have evolved into highly dedicated secretion systems that are structurally and functionally different (Thanassi and Hultgren, 2000). Therefore the secretion systems have been classified into different groups.

The Type I secretion systems (T1SS) are based on an assembly of an ATP-binding cassette (ABC) transporter protein within the inner membrane (Salmond and Reeves, 1993). A periplasmic protein connects the ABC transporter protein to an outer membrane protein that forms the secretion pore. In general the ABC transporters are dedicated to the transport of specific extra-cellular substrate proteins or toxins, like hemolysins.

The type II secretion systems (T2SS) are common among Gram negative bacteria (pathogenic and non-pathogenic) and represent the default machinery for protein secretion (Pugsley, 1993). Many of these substrate proteins and degrading enzymes are transported across the cytoplasmic membrane via the T2SS and are important for pathogenesis (Cianciotto, 2005). Typically, the T2SS appeared to be absent in representatives of at least 10 genera that belong to the γ-proteobacteria, including *S*. Typhi, *S.* Paratyphi and *S.* Typhimurium, after examination of sequences of Gram negative bacteria in the NCBI database (Cianciotto, 2005).

The type III secretion systems (T3SS) are complex bacterial protein assemblies that require at least 20 functional genes (Kubori *et al.*, 1998; Blocker *et al.*, 1999) (Figure 4). The gene clusters encoding T3SS are located on PAI (within *Salmonella* on Pathogenicity Island-1 and -2). The T3SS is assembled using different sub-units, of which many show similarities to the flagellum assembly machinery system. The main function of the T3SS is to translocate proteins across a third membrane, namely the host cell membrane. The primary purpose of the effector proteins secreted into the eukaryotic host cells is to interfere with the host cell functions (Kubori *et al.*, 1998; Blocker *et al.*, 1999).

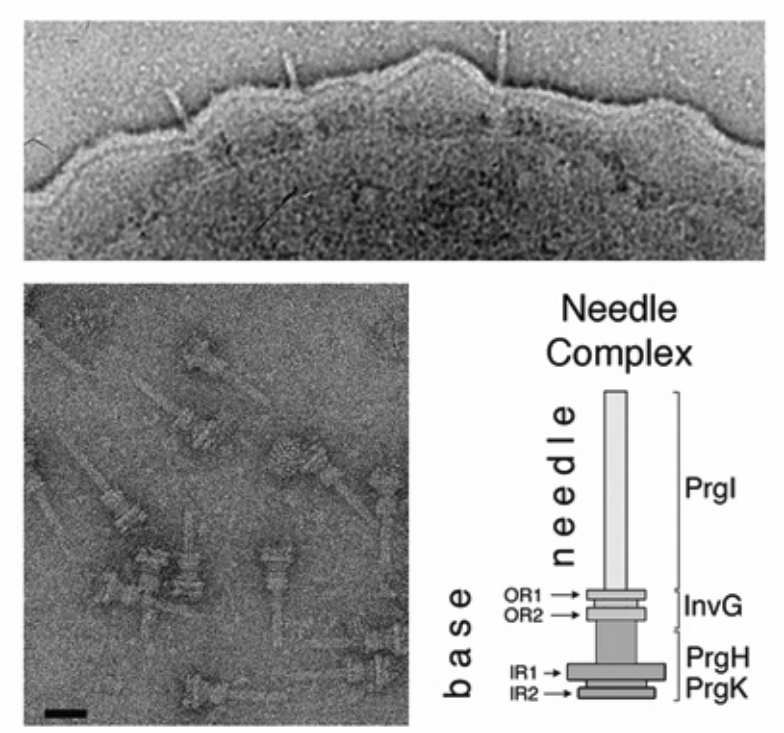

Figure 4. *"The type III secretion system: Electron micrograph of osmotically shocked S. typhimurium showing the needle complex embedded in the bacterial envelope (Upper) and released after detergent treatment (Lower left). Bar: 100 nm. Schematic representation of the* Salmonella *needle complex and its components (Lower right).PrgH, PrgK, and InvG make up the membrane embedded base structure, whereas PrgI forms the helical filament protruding into the extracellular environment. The inner rod anchors the filament into the base" (Marlovitz et al., 2004).*

The Type IV secretion systems (T4SS) are in many ways similar to the T3SS. The T4SS translocate proteins into eukaryotic cells and also have a complex structure build with at least 10 sub-units (Christie, 2001). The structure resembles conjugation systems that are able to translocate DNA. The genes encoding the T4SS are present in large gene clusters, most probably acquired as a complete PAI.

The Type V secretion systems (T5SS) are also referred to as autotransporters, since the complete transport system and the substrate protein are synthesized as one single pre-proprotein (Henderson *et al.*, 1998) which is secreted into the periplasm, cleaved and subsequently used to form a beta barrel structure in the outer membrane. The passenger domain of the proprotein passes through the pore of the beta-barrel. Finally, proteolytic cleavage allows the release of the passenger domain into the extra-cellular medium. The T5SS is encoded by PAI3 (SPI-3) in *S. enterica*.

Pathogenesis of plant / human pathogens

Several Gram-negative pathogens, like *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Erwinia spp*. and *Salmonella* serovars, have been recognized as capable of infecting both plants and humans (resp. Plotnikova *et al.*, 2000; Melnikov *et al.*, 2000; Iniguez *et al.*, 2005; Cao *et al.*, 2001). Much research has shown the existence of universal pathogenic mechanisms for many bacterial pathogens (Cao *et al.*, 2001). These include flagella and motility (Pallen *et al.*, 2005), lipopolysaccharides (LPS), exopolysaccharides (EPS), O-antigens, fimbriae, the ability to acquire iron, and resistance to oxidative stress (for review of these mechanisms; Toth *et al.*, 2003). Many of these pathogenicity determinants are secreted by a dedicated secretion system type (I to V) described above. However, one can expect large differences between plant pathogens and human pathogens, since mammalian host cells contain cell walls that are primarily lipid-based, whereas the outer membranes of plant cells are composed of polysaccharides (pectate and cellulose). Plant pathogenic bacteria contain essential genes coding for cell wall degrading enzymes that allow break down of these polysaccharides (Hugouvieux-Cotte-Pattat *et al.*, 1996; Toth *et al.*, 2003). These enzymes are sometimes also produced by human pathogenic bacteria, but to a much lesser extent. For example, a cellulase was isolated from *S.* Typhimurium that was able to degrade cellulosic substrates (Yoo *et al.*, 2004).

Also the T3SS is present in both bacterial plant pathogens and human pathogens (He, 2004). The T3SS in plant pathogens has a major role in manipulating plant defenses by translocation of effector proteins into the plant cytosol. Some effectors are also avirulence proteins that are recognized by the plant host resistance proteins. These trigger the hypersensitive response of the plant to restrict pathogen spread. Typically, the T3SS of plant pathogens resemble those of human pathogens with respect to the membrane-associated machinery. However, the extracellular components differ in that the human pathogens have a short needle-like structure, whereas the plant pathogens contain a longer, more flexible pilus (Romantschuk *et al.*, 2001). This can be explained by the fact that the plant pathogenic bacteria have to deliver the effector proteins into the host cytoplasm by first penetrating the plant cell wall. This in contrast to human pathogenic bacteria, which primarily need an intimate interaction with the host cell to enable translocation of protein via the T3SS.

Another important process shared by many bacteria is quorum sensing, which allows cell-to-cell communication. Quorum sensing is based on cell density-dependent regulation which in its turn is dependent on the environment or during pathogenesis (Miller and Bassler, 2001; Von Bodsman *et al.*, 2003). In plant pathogenic bacteria like *Erwinia* spp., quorum sensing

has been studied extensively (Miller and Bassler, 2001; Von Bodsman *et al.*, 2003). From these studies it is suggested that quorum sensing is linked to the regulation of cell wall degrading enzymes. If population densities have reached a level at which plant defenses can be overcome, the production of virulence factors is induced. Also, quorum sensing is suggested to be involved in root colonization and the regulation of conjugation between micro-organisms in the rhizosphere (Lugtenberg *et al.*, 2001). Most bacteria possess a quorum sensing synthase and its corresponding signal receptor. Typically, *Salmonella* serovars and *E. coli* possess the signal receptor but lack the signal generating enzyme gene. It was recently postulated that this might represent a mechanism for signal interception from other bacteria present in the close environment, while preventing a two-way communication (Ahmer, 2004).

Attachment of human pathogenic Salmonella serovars *to eukaryotic cells*

Prior to interaction with the host cell and subsequent invasion, bacteria have to move towards and attach to the cell wall or cell membrane first. In humans, movement to host cells is normally accommodated by the consumption of contaminated food. With plants, movement of bacteria towards the plant roots is initially based on chemotaxis. The ability of attachment to host cells is however dependent on surface-exposed bacterial organelles like fimbriae or pili (Darwin and Miller, 1999) and capsular polysaccharides (Eriksson de Rezende *et al.*, 2005).

At least four fimbriae have been genetically defined, giving namely type 1 fimbriae (Fim), plasmid-encoded fimbriae (PE), long polar fimbriae (LP) and thin aggregative fimbriae (curli). The type I fimbriae of *S. enterica* are closely related to those of *E. coli* and specifically bind to D-mannose receptors on different eukaryotic host cell types (Klemm and Krogfelt, 1994). The long polar fimbriae (LP) are not present in all Salmonellae; *S. typhi* and *S. arizoneae* appeared not to have acquired, or have lost the *lpf*-operon during evolution (Bäumler *et al.*, 1996[a]; Bäumler *et al.*, 1997). The LP fimbriae mediate adhesion to the cells of Peyer's patches (which are aggregations of lymphoid tissue that are usually found in the lowest portion of the small intestine in humans) (Bäumler *et al.*, 1996[b]). These LP fimbriae are suggested to contribute to pathogenicity in a sense that the fimbriae bring the bacterial cells close to the host cell facilitating the type III secretion system to invade the host cell.
Only four *Salmonella* serovars contain plasmids encoding fimbriae (PE), namely *S.* Typhimurium, *S.* Enteritidis, *S.* Choleraesuis and *S.* Paratyphi C. To what extent these PE fimbriae contribute to pathogenicity has not yet been resolved.

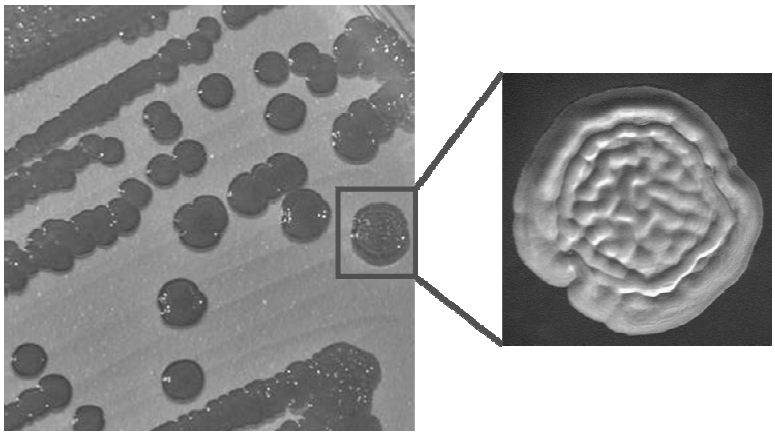

Figure 5. *Aggregative multicellular behaviour (rdar) of wild-type* Salmonella *Typhimurium grown at ambient temperature (below 28°C) on medium with low pH and low osmolarity due to cellulose production and curli (Barak et al., 2005).*

The last type of fimbriae is the thin aggregative fimbriae (curli), which were first identified and purified from *S.* Enteritidis (Collinson *et al.*, 1991). Curli-producing bacteria form a rigid multicellular network, called rdar (Römling *et al.*, 1998) (Figure 5). These are specifically formed at ambient temperatures (below 28°C), in rich media and low osmolarity.

The formation of curli is suggested to enhance the survival of bacterial cells in hostile environments (Collinson *et al.*, 1993) and mediate binding to, and invasion of, epithelial cells in *S. enterica*. Interestingly, these curli are also implicated in the attachment of *S. enterica* to alfalfa sprouts and are most likely also involved in attachment to roots of other plants (Barak *et al.*, 2005). Recently, also capsular polysaccharides were recognized to surround smooth and rugose types of *Salmonella* colonies enhancing attachment and biofilm formation (Eriksson de Rezende *et al.*, 2005).

The presence of at least four different fimbriae (and the capsular polysaccharides) suggests that the attachment of *Salmonella* serovars to host cells might be essential for the survival of *Salmonella* serovars in the environment. To what extent each of the systems contributes to the attachment to mammalian cells has not yet been elucidated, since in *in vitro* and *in vivo* studies one fimbriae system might compensate for another. Studies for attachment of *S. enterica* to plant cells have just started, and point to an important role of curli for attachment. For now, the role of the other fimbriae, polysaccharides or secretion systems is still unresolved. In any case, the presence of different fimbriae is essential to allow the

Salmonella cells to efficiently attach to host cells and to protect themselves from the hostile environment prior to invasion of the host cells.

Association of human pathogens with plants

To reduce the risk of food-borne illness caused by pathogens on produce to be consumed raw, much attention has been given to post-harvest surface disinfection methodologies. Yet, in recent years it became evident that certain human pathogens are not only able to attach to and proliferate on the surface of plant tissues (Lyytikianen *et al.*, 2004), but can also occur endophytically inside plant tissues (Kutter *et al.*, 2006). However, up to now only very few studies have investigated the molecular interaction between human bacterial pathogens and a plant host, and its physiological response. For *Arabidopsis thaliana* it was shown that the opportunistic human pathogen *Pseudomonas aeruginosa* PA14 attached to the leaf surface, congregated at the stomata or wounds, and then invaded the leaves and colonized the intercellular spaces (Plotnikova *et al.*, 2000). The bacterium was also able to make circular perforations in mesophyllic cell walls that allowed penetration. From this study it was concluded that *P. aeruginosa* PA14 is a facultative pathogen of *A. thaliana* that can cause local and systemic infection, eventually leading to plant death. Mutants of the human pathogenic *Staphylococcus aureus* (Prithiviraj *et al.*, 2005) that contained disrupted genes involved in animal pathogenesis, were attenuated in their ability to infect *A. thaliana*. This suggested that the same regulators that mediate synthesis of virulence factors essential for animal pathogenesis are also required for plant pathogenesis (Prithiviraj *et al.*, 2005). Resistance of *A. thaliana* to *S. aureus* was mediated by a direct effect of salicylic acid on the pathogen affecting attachment on the root surface and reducing pathogen virulence. In addition, *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 different *Salmonella* serovars were able to colonize *Medicago sativa* endophytically and epiphytically (Dong *et al.*, 2003). Also, an avirulent strain of *S*. Typhimurium colonized carrots and radishes which were grown on a field treated with contaminated manure composts or irrigation water (Islam *et al.*, 2004). Just recently, *S.* Typhimurium LT2 and DT104h were found to colonize barley sprouts endophytically during growth in an axenic system (Kutter *et al.*, 2006).
From these studies it is evident that human pathogens like *Salmonella* are able to colonize fresh produce endophytically and epiphytically and interact at a molecular level with the host plant.

Plant response to colonization by bacteria

During evolution plants have developed complex defense mechanisms to counter invasion of a broad range of pathogens. Plants respond to invasion by pathogens with a large set of biochemical and genetic changes (Noselli and Perrimon, 2000), including the production of reactive oxygen species (ROS), antimicrobial compounds, antioxidants and signaling molecules like salicylic acid (SA, jasmonic acid (JA) and ethylene (ET). These mechanisms respond also to local activation of programmed cell-death (based on a hyper-sensitivity response (HR)) and by the concurrent activation of cellular and molecular defenses, namely systemically acquired resistance (SAR) and induced systemic resistance (ISR).

Upon bacterial pathogen attack of incompatible plant cells, the plant responds in a hypersensitive manner leading to local necrosis, so that the invading bacteria are 'trapped' in these lesions (Dangl and Jones, 2001). Concurrently, the plant defense mechanism is triggered by the pathogen and the HR to induce SAR. Upon induction of SAR, plant parts that are not infected become more resistant to further infection with the pathogen (Ross, 1961). Next to this basic response, the colonization of roots with *Pseudomonas fluorescens* may trigger a plant-mediated resistance response in plant parts above ground to other pathogens. This second type of triggering the plant resistance is called ISR (Van Loon *et al.*, 1998). Biochemical mechanisms of ISR and SAR are different and the responses are additive in plant defense, as demonstrated by several studies. For example, SAR is effective against *Turnip crinkle virus*, but ISR is not. ISR is effective against *A. brassicicola*, whereas SAR is not (Ton *et al.*, 2002). The additive function of ISR and SAR was shown in the case of *P. syringae* pv *tomato* DC3000. The level of induced resistance was increased when both types of induced resistance were activated simultaneously (Van Wees *et al.*, 2000).

The signal transduction pathways of both systems are diverse. The triggering of SAR is expressed by a local and systemic increase of endogeneous levels of salicylic acid (SA) (Malamy *et al.*, 1990; Metraux *et al.*, 1990) and the concurrent up-regulation of a large set of genes, including the pathogenicity-related (PR) protein genes (Maleck *et al.*, 2000; Van Loon *et al.*, 1999; Ward *et al.*, 1991). ISR however, is independent of SA accumulation. From *Arabidopsis* mutants, namely jasmonic acid (JA)-response mutants, ethylene (ET)-response mutants and SAR-compromised mutants, it was revealed that the JA and ET response are required for triggering ISR (Knoester *et al.*, 1999; Pieterse *et al.*, 1998). Typically, both the ISR and SAR response are dependent on NPR1, a regulator of gene expression of plant defense pathways (Fan and Dong, 2002). But, the signaling pathway after NPR1 induction is different for both SAR and ISR. For SAR the PR-genes are activated (Pieterse *et al.*, 1996;

Van Wees *et al.*, 1997; Van Wees *et al.*, 1999), while ISR involves the concurrent induction or repression of genes related to JA and ET signaling (Pieterse *et al.*, 2002; Verhagen *et al.*, 2004).

Not only plant pathogens induce defense responses in plants. Recently some evidence was obtained that human pathogenic bacteria also induce defense responses of plants upon colonization. In *Arabidopsis*, colonization and invasion of the human opportunistic pathogen *P. aeruginosa* PA14 in leaf tissues resulted in maceration and autolysis of plant cells, eventually leading to soft rot symptoms (Plotnikova *et al.*, 2000). An *Arabidopsis* npr-1-1 mutant supported a higher level of *P. aeruginosa* PA14 growth than wild-type plants (Volko *et al.*, 1998). Since this mutant failed to express the PR1-gene, these results imply that the plant reacts to colonization by the pathogen in a defensive manner. For *S. aureus* it was found that the pathogenicity on *A. thaliana* was mediated by a direct effect of SA on *S. aureus* affecting the attachment and aggregate formation on root surfaces, or by SA-dependent host responses (Prithiviraj *et al.*, 2005). Another study focused on the endophytic colonization of *Medicago truncatula* by *S.* Typhimurium (Iniguez *et al.*, 2005). From a set of mutants there was evidence that the flagella primarily induce the SA-independent pathway, whereas effectors of the Type III secretion system of SPI-1 induce the SA-dependent defense pathway. A *S.* Typhimurium mutant defective in the type III secretion system also failed to activate the PR1-gene of *Medicago* spp., giving a strong indication for the assumption that colonization of plants, i.e. *Medicago* spp., by *Salmonella* serovars is countered by both SA-independent and -dependent defense systems.

These first studies for plant responses to colonization by human pathogenic bacteria strongly point to the ability of human pathogens to colonize plants and to induce plant defense systems. However, which virulence factors are essential for *Salmonella* serovars to allow endophytic colonization of a plant, what role the differences in plant susceptibility or environmental factors play, has not yet been elucidated. Insight in the interaction between human pathogens and a plant would allow the development of preventive treatments to eventually reduce the risk for human health problems.

Scope of this thesis

Contamination of plants with human pathogens is gaining intensive attention due to the increase of outbreaks related to consumption of fresh vegetables. From all produce-associated outbreaks the most commonly found pathogen is *Salmonella*, followed by *E. coli*

O157:H7, which can incite severe illness or even death. Critical points for introduction of these human pathogens in the production chain are mainly thought to be the use of manure or compost for fertilization and the use of contaminated water on crop fields. To understand the route of infection, and to identify factors influencing the infection rate, the transmission of the pathogens from manure or compost to soil into the plants needs to be determined. Highly sensitive and specific quantitative detection methods are required since these pathogens are surrounded by a great variety of micro-organisms in the substrates of interest (manure, compost and soil). These methods should be robust in a sense that inhibiting agents are omitted or countered during the DNA extraction or amplification procedure. Eventually the procedural efficiency is of large importance for reliable quantification and should therefore be controlled.

In addition, the interaction between the human pathogen and the plant is thought to play a major role in infection efficiency and colonization of the plant. The basis of the interaction is believed to be embedded in the genomes of both organisms, mainly the expression of so-called virulence genes of the pathogens, and defense mechanisms of the plant. Research in this area would determine if human pathogens behave like a plant pathogen, a parasite, or act for example as symbionts in the plant. Each lifestyle is dependent on presence or absence of genes being expressed or suppressed by either the pathogen or plant. Insight in the fundamental pathways and interactions between a human pathogen and plant might eventually lead towards the prevention of human pathogens to be introduced in the production chain of freshly consumed vegetables.

After the development of molecular detection methods for both *S. enterica* and *E. coli* O157:H7, the research for this thesis has mainly focused on *S. enterica*, since it was found more often as the causal agent of produce-related outbreaks than *E. coli* O157:H7.
To study the intimate interaction between the plant host and the human pathogen, *Lactuca sativa* (lettuce) was selected as plant host due to its economic importance and strong association with foodborne outbreaks. Thus, in this thesis *Salmonella* in association with *Lactuca sativa* (lettuce) was selected as model system to identify factors related to, or important for, colonization of a plant by a bacterial human pathogen.

Chapter 1 provides an overview of recent outbreaks of enteric diseases, in particular Salmonellosis, related to the consumption of fresh produce, describes the currently available detection methods for enteric pathogens, and reviews the literature on the taxonomy and pathogenesis of *Salmonella*.
Chapter 2 describes a comparison of real-time PCR methods for detection of *Salmonella*

enterica and *Escherichia coli* O157:H7, and development of a general internal amplification control

Chapter 3 presents a comparison of methods of extraction of *Salmonella enterica* serovar Enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control

Chapter 4 involves the physiological and molecular response of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin

Chapter 5 describes the differential interaction of human pathogenic *Salmonella enterica* serovars with lettuce cultivars and factors contributing to colonization of lettuce

Chapter 6 provides a general discussion based on the research and results of this thesis. The detection of *Salmonella* serovars, their association with lettuce, and the microbe-host factors influencing the efficiency of epiphytic and endophytic colonization are discussed.

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Chapter 2

Comparison of real-time PCR methods for detection of Salmonella enterica *and* Escherichia coli *O157:H7, and introduction of a general internal amplification control*

Journal of Microbiological Methods (2004) **59:**337-349

Comparison of real-time PCR methods for detection of *Salmonella enterica* and *Escherichia coli* O157:H7, and introduction of a general internal amplification control

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Summary

The objectives of this study were to compare different real-time PCR-based methods for detection of either *Salmonella enterica* or *Escherichia coli* O157:H7 with respect to sensitivity, precision and accuracy. In addition, a general internal amplification control (IAC) is presented, allowing prevention of false negative results. The IAC allows insight in amplification efficiency and enables a more accurate quantification with the evaluated real-time PCR methods. Implementation of the IAC with the different PCR methods did not affect the precision of the methods, but the sensitivity was reduced 10-fold. Introduction of an IAC with the *S. enterica* specific detection method showed a shift in Ct-value (increase of target Ct-value with 0.45 ± 0.17 cycles), while with the method to detect *E. coli* O157:H7 no influence of IAC co-amplification was observed. The quantification threshold of the methods in which the IAC was included, was determined at 1 pg of target DNA (equal to 200 CFU) per reaction. Qualitative detection was feasible down to 10 fg of target DNA per reaction using both methods in which the IAC was incorporated. The adjusted methods have the potential to provide fast and sensitive detection of *S. enterica* or *E. coli* O157:H7, enabling accurate quantification and preventing false negative results by using the general IAC.

Introduction

In recent years the occurrence of health threatening pathogens in the food production chain has been of major concern. Many food-borne outbreaks have been associated with *Salmonella enterica* or *Escherichia coli* O157:H7 contaminated consumables, like beef, pork, poultry and vegetables (Mead *et al*., 1999; Todd, 1989; Swinbanks, 1996; Bradbury, 1997; Hilborn *et al.*, 1999; Ackers *et al*., 1998; Viswanathan and Kaur, 2001). Infection with enterohaemorrhagic *E. coli* O157:H7 (EHEC) can cause a wide range of disease symptoms like haemorrhagic colitis, haemolytic-uremic syndrome (HUS), mild to bloody diarrhea, and thrombotic thrombocytopenic purpura in humans (Karmali *et al*., 1985; Riley *et al*. 1983). *Salmonella* spp. can infect a broad host spectrum, including mammals, birds and reptiles, and is associated with acute gastro-intestinal illnesses around the world (Baird-Parker, 1990).

Until now, standardized diagnostic procedures to detect the presence of these pathogens in food samples are based on microbiological culturing methods (*E. coli* O157:H7; ISO 16654/2001, *S. enterica*; ISO 6579/2002), requiring up to five days until results are obtained (Stewart *et al*., 1998). In order to reduce the time demand, alternative techniques like immunological assays (Acheson *et al*., 1994; Bolton *et al*., 2000) and molecular methods (Bej *et al*., 1994; Heller *et al*., 2003; De Medici *et al.*, 2003) have been applied to detect *S. enterica* or *E. coli* O157:H7 from various samples.
Especially real-time PCR methods such as 5' nuclease Taqman PCR (Holland *et al*., 1991) and PCR combined with molecular beacons (Tyagi *et al*., 1996), have shown promising results due to the rapid, sensitive and specific detection of pathogens (Higgins *et al*., 1998; Bassler *et al*., 1995).

To detect *S. enterica* and *E. coli* O157:H7, real-time PCR methods have been reported based on the amplification of species-specific genes, or genes related to pathogenicity or virulence. Recently, the detection of *S. enterica* was accomplished by real-time Taqman PCR methods directed to the *spaQ*-gene (Kurowski *et al*., 2002), the *invA*-gene (Hoorfar *et al*., 2000) and a molecular beacon-based method directed to the *himA*-gene (Chen *et al*., 2000). Also for the detection of *E. coli* O157:H7 real-time Taqman PCR methods have been described, directed to the *rfbE*-gene (Fortin *et al*., 2001) and, in a simultaneous matter, the three genes Shiga toxin 1 (*stx1*-gene), Shiga toxin 2 (*stx2*-gene) and intimin (*eae*-gene) (Molecular beacon-based detection, Fortin *et al*., 2001; Taqman-based detection, Sharma *et al*., 1999 and Ibekwe *et al.*, 2002). Each of these methods was sensitive and specific, but

these methods have not yet been compared with respect to sensitivity, precision (determined by the standard deviation among replicates of one sample) and accuracy (considered to be the coefficient of determination for regression of Ct-values versus log [amount of target DNA] for each PCR method compared to the other methods).

Real-time PCR methods are often used for quantification of initial target DNA. Unfortunately, amplification efficiencies can be different from sample to sample due to the effects caused by inhibition of amplification, human failures or preparation errors. This implies that quantification, even with external controls, does not always represent a correct calculation of initial amount of target in each sample. To eliminate part of these drawbacks, different approaches of using an internal amplification control in each real-time PCR have been described recently (i.e. Hoorfar *et al*., 2000; Raggam *et al*., 2002; Savli *et al*., 2003; Vandesompele *et al*., 2002). In this paper an alternative approach is presented using different primer sets for amplification of the target DNA and the internal amplification control (IAC) DNA. The latter is directed towards the amplification of a *gfp*-coding sequence (Prasher *et al.*, 1992), originally derived from an organism not found in natural environmental substrates (except in the cnidarian jellyfish *Aequorea victoria*). Due to these features, the IAC described in this paper is particularly suitable as a general IAC, which can be used irrespective of the substrate or target organism to be detected.

In this study, a comparison of three real-time PCR methods for the detection of each *S. enterica* and *E. coli* O157:H7 was performed. The applicability of each method was determined with respect to sensitivity, precision and accuracy. Secondly, a general internal amplification control was developed to allow elimination of false negative results. Its influence on the sensitivity, precision and accuracy of each method was evaluated. Finally, the applicability of quantification of initial target DNA using the real-time methods, including the IAC, was investigated and both the quantification threshold and detection limit were evaluated.

Materials and Methods

Bacterial strains and DNA extraction

A suspension of *S.* Enteritidis ATCC 13076, grown overnight in tryptic soy broth (late log phase) at 30°C was kindly provided by RIKILT, The Netherlands. A bacterial culture of *Escherichia coli* O157:H7, strain 102, grown overnight in Luria broth (LB)-medium (late log

phase) at 37°C was kindly provided by RIVM, The Netherlands. A bacterial culture of a genetically modified *S.* Typhimurium strain 110, harboring a green fluorescent protein (*gfp*)-coding sequence within the genome was kindly provided by U. Römling (Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden). A culture of genetically modified *E. coli* O157:H7 (strain B6-914 *gfp*-91) containing a *gfp*-construct (Fratamico *et al*., 1998; Dykes *et al*., 2001) was kindly provided by Fratamico. These strains were grown on LB medium to late log phase before use.

DNA extraction was performed as follows: first 3 ml bacterial suspension of each culture (approx. 10^9 cells / ml) was centrifuged (5000 rpm for 10 min). Subsequently, the pellet was re-suspended in chaotropic AP1-buffer of the DNeasy DNA extraction kit (Qiagen, Westburg, Germany). DNA extraction was further performed according to the protocol supplied by the manufacturer, including the Qia-shredder to discard large particles. The bacterial DNA was recovered in 100 µl of elution buffer supplied with the DNeasy DNA extraction kit. To determine the amount of DNA, the optical density of a ten times diluted fraction of each DNA eluate was measured using the 260nm / 280nm ratio calculation. Since the genome of *S.* Enteritidis (ATCC 13076) DNA and *Escherichia coli* O157:H7 (strain 102) is 5 Mbase and 5.5 Mbase, respectively, 1 CFU equals respectively 5.4 and 5.9 femtogram of double stranded genomic DNA.

Primers and probes

Sequences of primers and probes for detection of *S. enterica* were derived from Chen *et al*., (2000), Hoorfar *et al*., (2000) and Kurowski *et al*., (2002). For the detection of *E. coli* O157:H7 the primers and probes were derived from Fortin *et al*., (2001), Ibekwe *et al*., (2002) and Sharma *et al*., (1999). In this study the molecular beacons were labeled with 6-carboxyfluorescein (FAM). Taqman probes were labeled with 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET) or 6-carboxytetramethyl-rhodamine (TAMRA). Both FAM and TET colored probes were labeled with an Eclipse Dark Quencher at the 3' end, the TAMRA colored probe was labeled at the 3' end with Ellequencher (Eurogentec, Maastricht, The Netherlands), to ensure a maximum reduction of background fluorescence.

For the development of an IAC for real-time PCR, the primers FP_{GFP} (5' TGG.CCC.TGT.CCT.TTT.ACC.AG 3') and RP_{GFP} (5' TTT.TCG.TTG.GGA.TCT.TTC.GAA 3') were designed, based on the DNA sequence of the *gfp*-coding gene (Prasher *et al*., 1992, accession number: P42212). This gene is present in both *S.* Typhimurium strain 110-*gfp* and *E. coli* O157:H7 strain B6-914 *gfp*-91. The primers flanked a sequence of 31 nucleotides, resulting in an amplification product of 72 nucleotides.

The Taqman probe PYY_{GFP} (5' AAC.CAT.TAC.CTG.TCC.ACA.CAA.TCT.GCC.C 3') was designed to specifically hybridize to the *gfp*-coding DNA at 55°C up to 60°C, and was labeled at the 5' end with Yakima Yellow (YY; Eurogentec) and at the 3'end with an Eclips Dark Quencher (Eurogentec).

Preparation of DNA dilution series

DNA was extracted in duplicate from liquid bacterial cultures containing either *S.* Enteritidis ATCC 13076 or *E.coli* O157:H7 (strain 102). Subsequently, from each DNA extract a 10-fold dilution series of target DNA was prepared in duplicate. Each series ranged from 400 pg DNA / µl down to 400 ag DNA / µl, and included a negative control (containing no target DNA).

To obtain internal amplification control DNA, total DNA was extracted from liquid culture of the *S.* Typhimurium strain 110 containing the *gfp*-gene (single-copy, present in genome) and the *E. coli* O157:H7 strain B6-914 *gfp*-91 containing the *gfp*-gene (multicopy, present in plasmid). A 10-fold dilution series ranging from 400 pg DNA / µl down to 400 ag DNA / µl and including a negative control, was prepared in duplicate.

Real-time PCR

Three real-time PCR methods as described for detection of *S. enterica* (Chen *et al*., (2000), method $Salm_{MB\text{-}himA}$; Hoorfar *et al*., (2000), method $Salm_{Taq\text{-}invA}$; Kurowski *et al*., (2002), method $Salm_{Taq\text{-}spaQ}$) were tested. Also three real-time PCR methods as described for the detection of *E. coli* O157:H7 (Fortin *et al*., (2001), method $Ecol_{MB\text{-}rfbE}$; Ibekwe *et al*., (2002), method $Ecol1_{Taq\text{-}sse}$; Sharma *et al*., (1999), method $Ecol2_{Taq\text{-}sse}$) were tested. All methods were slightly modified regarding the annealing temperature and elongation time, herewith improving the sensitivity of the methods. In each case (except the method $Ecol1_{Taq\text{-}sse}$) the initial 10-min incubation at 95°C was followed by 40 cycles. Each cycle consisted of 15 sec incubation at 95°C and an annealing temperature of 57°C for 60 sec ($Salm_{MB\text{-}himA}$), 55°C for 60 sec ($Salm_{Taq\text{-}invA}$), 60°C for 60 sec ($Salm_{Taq\text{-}spaQ}$), 41°C for 30 sec followed by 60°C for 45 sec ($Ecol_{MB\text{-}rfbE}$) or 60°C for 60 sec ($Ecol2_{Taq\text{-}sse}$). The PCR method $Ecol1_{Taq\text{-}sse}$ used 50 cycles, of which each cycle (after initial incubation at 95°C for 10 min) consisted of respectively 95°C for 20 sec, 55°C for 30 sec and 72°C for 40 sec incubation.

PCR was performed using the components of a real-time PCR kit (qPCR core kit; Eurogentec). Each PCR consisted of 3 µl 10x Reaction buffer containing ROX [5-(and -6)-carboxy-X-rhodamine], 1.2µl dNTP solution (5mM of each dNTP, including dUTP), 3 µl of 50 mM $MgCl_2$, 1.8 µl of 5 µM of each primer, 1.2 µl of 5 µM of each Taqman probe or molecular

beacon, and 0.15 µl 5U/µl Hot Goldstar DNA polymerase. Water was added to a volume of 27.5 µl per reaction. Before PCR was started, 2.5 µl of target DNA was added to each reaction. For each method the fluorescence increase was measured in real-time during amplification using an ABI 7700 sequence detector (Applied Biosystems, Foster City, USA). To analyze the PCR data, the threshold (the minimal fluorescence above which a sample is determined positive) was calculated by taking the average of the fluorescence of the negative controls (40 measure points per control x 12 controls, n=480) plus four times the standard deviation (S.D.) of the fluorescence of the negative controls (p=0.99). Subsequently the threshold was used to determine the Ct-value for each PCR sample from each PCR method.

Real-time PCR including the Internal Amplification Control

The PCR protocol of the IAC consisted of an initial incubation of 95°C for 10 min, followed by 40 cycles of incubation for 95°C for 15 sec and 60°C for 60 sec, respectively. For implementation of the IAC with both real-time PCR methods for detection of respectively *S. enterica* and *E. coli* O157:H7, to each reaction 0.45 µl of 5µM of each of the primers FP_{GFP} and RP_{GFP}, and 0.3 µl of 5 µM of the probe PYY_{GFP} was added. Water was added to a volume of 25 µl. Prior to PCR, 2.5 µl of IAC DNA and 2.5 µl of target DNA was added. For the assay to detect *S. enterica*, the IAC DNA suspension consisted of DNA from the genetically modified *E. coli* O157:H7 strain B6-914 *gfp*-91 (Fratamico *et al*., 1998). For detection of *E. coli* O157:H7, the IAC DNA originated from the genetically modified *S.* Typhimurium strain 110-*gfp*.

Implementation of the internal amplification control

To test the influence of the amount of IAC DNA on target amplification with respect to the sensitivity, an experimental matrix was prepared in triplicate. Four different amounts of IAC DNA (*S.* Typhimurium-*gfp* DNA: 10 pg / 1 pg / 100 fg / 0 fg per PCR; *E. coli* O157:H7-*gfp* DNA: 100 fg / 10 fg / 1 fg / 0 fg per PCR) versus a 10-fold dilution series (400 pg DNA down to 400 ag DNA/µl, and negative control) of target DNA (respectively *E. coli* O157:H7 DNA and *S*. Enteritidis ATCC 13076 DNA) were tested separately using the detection methods $Salm_{Taq\text{-}invA}$ and $Ecol_{Taq\text{-}sse}$. To evaluate the applicability for quantification of the method $Salm_{Taq\text{-}invA}$ including the most optimal amount of IAC, a 10-fold dilution series (400 pg DNA down to 400 ag DNA/µl, and negative control) of *S.* Enteritidis ATCC 13076 DNA was tested in twelve-fold.

Statistical analysis

The sensitivity was considered to be the highest Ct-value, i.e. the highest number of PCR cycles still resulting in a detectable fluorescence signal above the threshold, defined by the mean plus four times the S.D. of the fluorescence signal of 4 control samples (99% confidence). Sensitivities of the methods could not be compared statistically, since the number of positive samples at the highest Ct value varied from test to test, and (naturally) the S.D. of the Ct-values were high at the lowest amount of target DNA.

The precision was considered equal to the S.D. of the Ct-values among replicates for each method and amount of target DNA added to a sample. These S.D. were determined for *S.*Enteritidis DNA or *E. coli* O157:H7 DNA in four dilution series (tested in triplicate). The average S.D. per amount of DNA was plotted versus the log [amount target DNA per 30 µl of PCR solution] for each method separately. The precision estimates were compared among detection methods using ANOVA (SPSS, Inc., Chicago, Illinois 60606, US).

The accuracy of each method was estimated as the coefficient of determination for linear regression of the average Ct-values versus the log [amount of target DNA per 30 µl of PCR solution] per dilution series. The accuracy's (of four dilution series) were statistically compared (ANOVA) among PCR methods.

Each of the most promising PCR methods without IAC was compared with its counterpart with IAC using paired t-tests (SPSS). Next, the Ct-value of each PCR without IAC was subtracted from that with IAC for each amount of target DNA tested to determine the influence of the IAC amplification on Ct-values of the target DNA. The averages and S.D. of these differences were calculated for each amount of target DNA. These S.D. and means were used to determine the minimal amount of target DNA above which the influence of IAC co-amplification on Ct-values of target DNA was constant (with acceptably low S.D.).

Finally, a calibration line was produced for the $Salm_{Taq\text{-}invA}$ detection method including the appropriate IAC by regressing mean Ct-values (n=12) on log [amount of target DNA], and 95% confidence limits were calculated for each mean value.

Results

Comparison of real-time PCR methods to detect S. enterica

The compared real-time PCR methods ($Salm_{MB\text{-}himA}$, $Salm_{Taq\text{-}invA}$, $Salm_{Taq\text{-}spaQ}$) differed in sensitivity (Fig. 1). $Salm_{Taq\text{-}invA}$ and $Salm_{Taq\text{-}spaQ}$ had the lowest detection limit of 1 fg of DNA / PCR with 5 out of 12 and 2 out of 12 reactions positive, respectively. $Salm_{MB\text{-}himA}$ was clearly less sensitive with a detection limit or 10 fg of DNA (5 out of 12 positive). There was no variation in sensitivity among replicate series for each of the methods.

The precision of each method was displayed by plotting the average S.D. per amount of DNA versus the log [target DNA amount added to PCR] (Figure 1). When target DNA amounts of 100 fg / PCR or higher were used, a high precision within replicates was observed (average S.D. < 0.63 cycles), while with target DNA amounts of 1-10 fg / PCR higher variances (average S.D. of 2.05 to 2.47 cycles) were noticed (Figure 1). The accuracy of each method, as determined by linear regression analysis of the average of Ct-values versus the log [amount of DNA] per dilution series, was very high (Table 1). The R^2 values did not differ among methods (ANOVA, significance of 0.673). Based on sensitivity, precision and accuracy, the method $Salm_{Taq\text{-}invA}$ was selected and used for the following experiments.

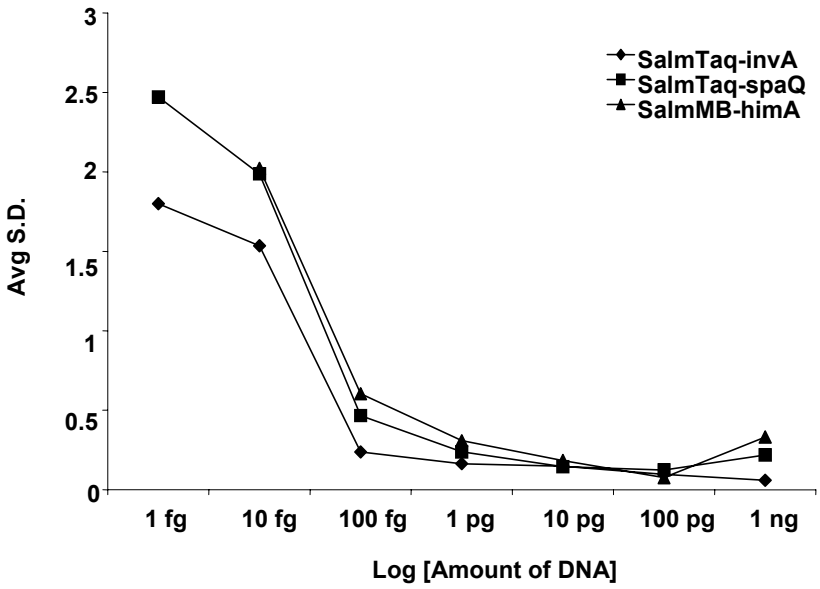


Figure 1. *The average of S.D. plotted as function of the initial amount of* S. *Enteritidis ATCC13076 DNA. Each dilution series was tested in triplicate. Each measure point represents the average S.D. of Ct-values from one amount of DNA from all dilution series.*

Comparison of real-time PCR methods to detect E. coli O157:H7

To detect *E. coli* O157:H7, the method Ecol1$_{Taq\text{-}sse}$ appeared at least 100-fold less sensitive during initial experiments and required at least 50 PCR cycles (results not shown) in contrast to the other two methods (40 PCR cycles) and was therefore not evaluated further.

The sensitivity of the two other methods was 1 fg of DNA / PCR using Ecol2$_{Taq\text{-}sse}$ (4 out of 12 positive) directed to the *stx1*-gene (FAM-fluorescence) and *stx2*-gene (TET-fluorescence), and 10 fg of DNA using Ecol$_{MB\text{-}rfbE}$ (10 out of 12 positive) (Figure 2). In case of detection using FAM or TET fluorescence, the precision was high (S.D. < 0.77 cycles) when 10 fg of DNA (Ecol$_{MB\text{-}rfbE}$) or 100 fg of DNA (Ecol2$_{Taq\text{-}sse}$) / PCR, or more, was used (Figure 2). The precision was very low (up to an average S.D. of 2.59 cycles) when detecting the *eae-gene* using TAMRA fluorescence (Ecol2$_{Taq\text{-}sse}$). The accuracy of both methods, as determined by linear regression analysis of the average Ct-value versus the log [amount of DNA] per dilution series, was high (Table 1).

Table 1. *Coefficients of determination (R^2) for linear regression of Ct-values (number of cycles) on amount of DNA added (400 ag to 400 pg per µl) obtained with 3 different real-time PCR methods to detect* Salmonella enterica *and 4 different real-time PCR methods to detect* E. coli *O157:H7 carried out in triplicate.*

Method	***S*. Enteritidis ATCC 13076 DNA**					
	R^2-value				Mean	S.D.
	Series 1*	Series 2	Series 3	Series 4		
Salm$_{MB\text{-}himA}$	0.9958	0.9868	0.9929	0.9963	0.99295	0.004365
Salm$_{Taq\text{-}invA}$	0.9994	0.9978	0.9955	0.993	0.99645	0.002789
Salm$_{Taq\text{-}spaQ}$	0.9952	0.9983	0.9789	0.9993	0.99293	0.009512

	***E. coli O157:H7* strain 102 DNA**					
	R^2-value				Mean	S.D.
	Series 1	Series 2	Series 3	Series 4		
Ecol$_{MB\text{-}rfbE}$	0.9990	0.9949	0.9997	0.9975	0.99778	0.002125
Ecol2$_{Taq\text{-}sse}$ *stx1*-gene	0.9939	0.9989	0.9983	0.9917	0.99570	0.003476
Ecol2$_{Taq\text{-}sse}$ *stx2*-gene	0.9964	0.9982	0.9976	0.9993	0.99788	0.001209
Ecol2$_{Taq\text{-}sse}$ *eae*-gene	0.9827	0.9829	0.9818	0.9924	0.98495	0.004990

**Dilution series of bacterial DNA from* Salmonella *Enteritidis ATCC 13076 or* Escherichia coli *O157:H7 strain 102, obtained after nucleic acid extraction*

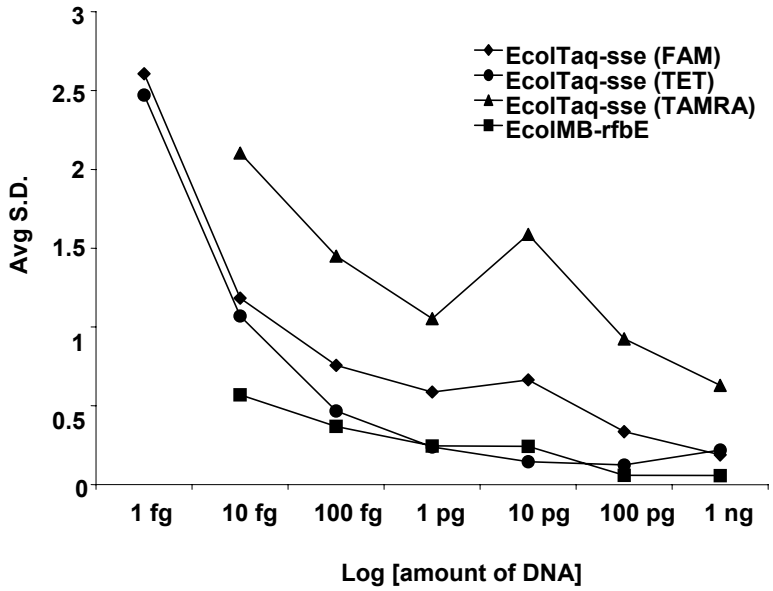


Figure 2. *The average of S.D. of Ct-values plotted as function of the initial amount of* E. coli *O157:H7 (strain 102) DNA. Each dilution series was tested in triplicate. Each point presents the average S.D. of Ct-values from one amount of DNA from all dilution series.*

The R^2-values did not differ among the tested methods (ANOVA significance of 0.785), except for detection of the *eae-gene* using TAMRA fluorescence, which had a significantly lower R^2 than the other fluorophores of the same method (Table 1). Based on these results the methods $Ecol_{MB\text{-}rfbE}$ and $Ecol2_{Taq\text{-}sse}$ were selected for further evaluation.

Development of a real-time PCR internal amplification control

An internal amplification control (IAC) was developed to detect specifically the presence of a *gfp*-coding sequence. Primers and probe were designed such to avoid cross-reactivity with the methods for detection of *S.* Enteritidis or *E. coli* O157:H7. A detection limit of 1 fg of DNA from the *E. coli* O157:H7 *gfp*-strain (not shown) and 100 fg of DNA from *S.* Typhimurium *gfp*-strain (Figure 3) was obtained. When testing specificity of the IAC, the *E. coli* O157:H7-specific method $Ecol_{MB\text{-}rfbE}$ with addition of *S.* Typhimurium-*gfp* DNA unexpectedly showed a fluorescence increase during the final cycles of amplification (not shown). Therefore the *E. coli* O157:H7-specific method $Ecol_{MB\text{-}rfbE}$ was not evaluated further with the IAC. In all following experiments the *S.* Typhimurium-*gfp* DNA (IAC_S) was used with the *E. coli* O157:H7 detection method $Ecol2_{Taq\text{-}sse}$, and the *E. coli* O157:H7-*gfp* DNA (IAC_E) was used in the PCR method specific for *S. enterica* detection ($Salm_{Taq\text{-}invA}$).

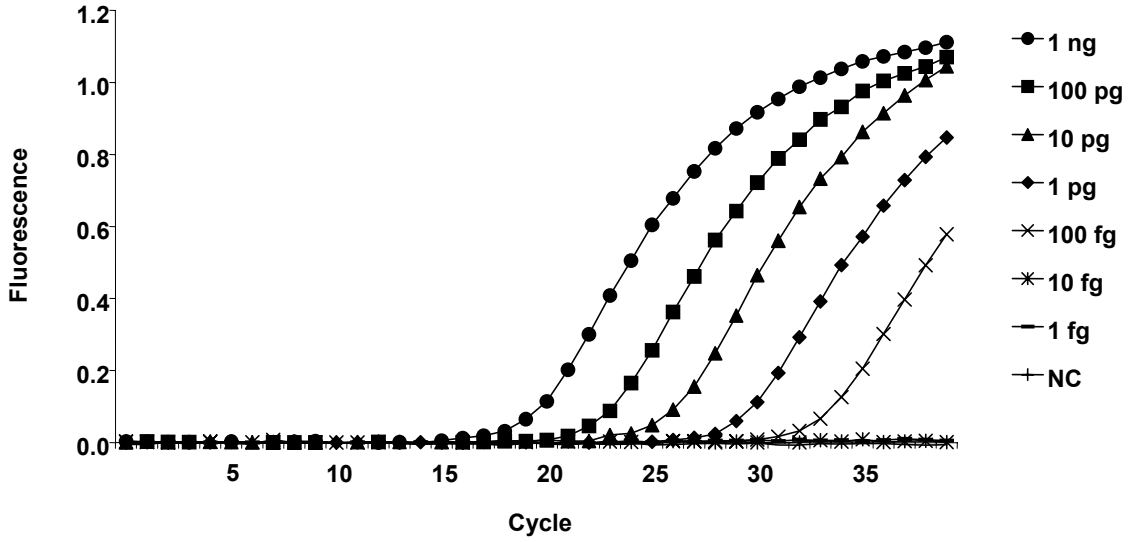


Figure 3. *Real-time amplification and detection of a 10-fold dilution series of* Salmonella *Typhimurium*-gfp *DNA using the IAC Taqman PCR. The fluorescence is plotted versus the cycle-number of PCR.*

Implementation of the internal amplification control

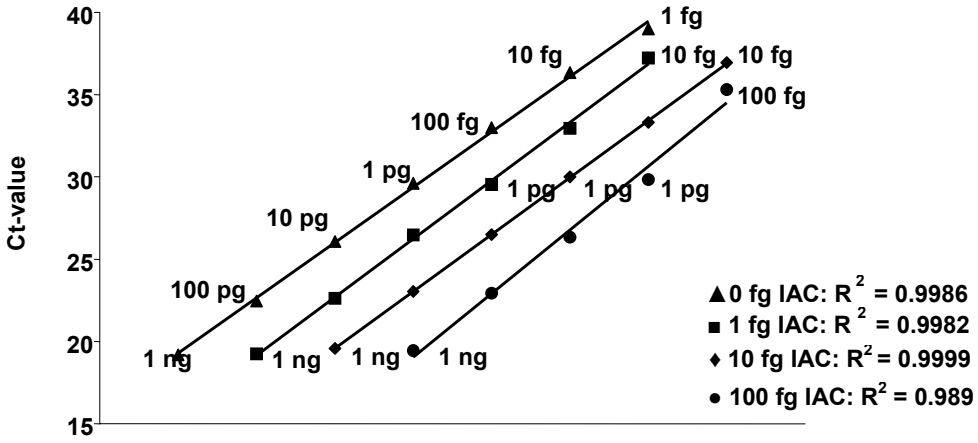
Irrespective of the method used, an increase in the amount of IAC DNA led to an increase of the lowest detectable amount (i.e. less sensitive) of target DNA (Figure 4A and B). In all samples the IAC_E was clearly detected (YY fluorescence), regardless the amount of *S. enteritidis* DNA (FAM fluorescence). In case of *E. coli* O157:H7 DNA detection, higher amounts (10 pg or higher / PCR) of *E. coli* O157:H7 DNA resulted in major suppression of the IAC_S amplification (results not shown). With co-amplification of IAC_E, the lowest detection limit of PCR for detection of *S.* Enteritidis (10 fg / PCR) was obtained when using 10 fg of IAC_E DNA / PCR (IAC_E average Ct-value of 31 cycles) (Figure 4A). With co-amplification of IAC_S the lowest detection limit of PCR for detection of *E. coli* O157:H7 *stx1*-gene and *stx2*-gene (10 fg / PCR) was obtained using 1 pg of IAC_S DNA / PCR (IAC_S average Ct-value of 34.7 cycles) (detection *stx1*-gene: figure 4B).

Influence of internal positive control on target amplification

With respect to detection of *S.* Enteritidis*,* the dilution series of target DNA with co-amplification of 10 fg of IAC_E DNA / PCR, and without co-amplification of IAC_E DNA showed a significant difference between the Ct-values (t-value: 5.55, critical t-value: 1.94, when α=0.05 and 6 degrees of freedom) (Table 2). The difference between the Ct-values (with and

without IAC_E amplification) was calculated for each amount of target DNA (Table 2). Both the

A: *S. enterica*: $Salm_{Taq\text{-}invA}$;



B: *E. coli* O157:H7: $Ecol2_{Taq\text{-}sse}$; *stx1*-gene (FAM) detection

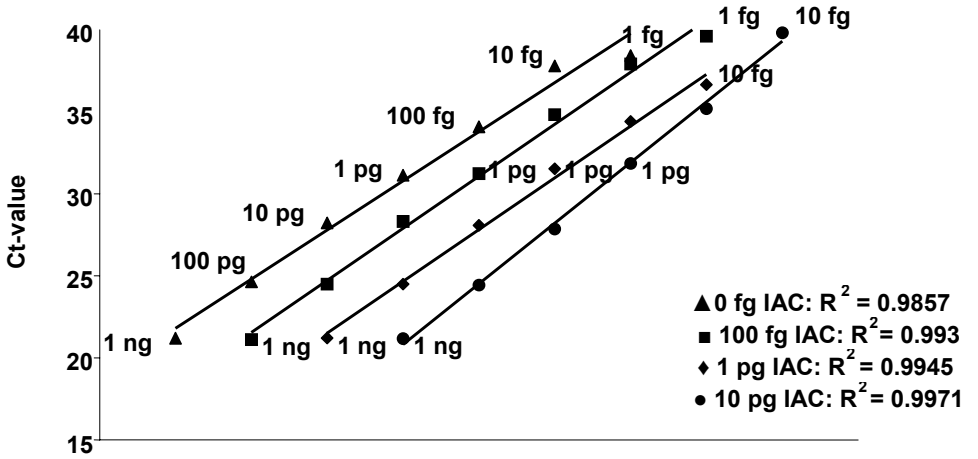


Figure 4. *Linear regression analysis of Ct-values in the presence of different amounts of internal amplification control (IAC), versus the amount of detectable target DNA added / PCR (A, method $Salm_{Taq\text{-}invA}$; B, method $Ecol2_{Taq\text{-}sse}$). Each combination was tested in triplicate. Each point represents the average of Ct-values measured from the same amount of target DNA : IAC DNA combination.*

overall average of these differences (0.58 cycles) and the associated average S.D. (± 0.5cycles) increased too much to determine the exact extent of influence of IAC amplification on *S.* Enteritidis DNA amplification. Amounts of *S.* Enteritidis DNA of 100 fg or lower / PCR indicated an influence of the IAC_E co-amplification with a S.D. of at least 1.03 cycles (Table

2), making quantification in this lower detection region not valid.

Table 2. *Differences in Ct values (cycles) between Taqman tests for detection of* S. enterica *and* E. coli *O157:H7 with and without internal amplification control (IAC), the standard deviations of these differences, and t-test results to determine the magnitude of the correction factor of Ct-values (cycles)*

	S. enterica		***E. coli* O157:H7**			
			Stx1-gene		*Stx2*-gene	
	Cycles[b]		Cycles		Cycles	
Amount of target[a]	(+IAC) - (-IAC)	S.D.[c]	(+IAC) - (-IAC)	S.D.	(+IAC) - (-IAC)	S.D.
1 ng	0.39	0.150776	0.02	0.27453	-0.67	0.662369
100 pg	0.59	0.032146	-0.14	0.219545	-0.34	0.244199
10 pg	0.42	0.069041	-0.14	0.173013	-0.35	0.520705
1 pg	0.39	0.411096	0.39	0.224648	-0.50	0.228327
100 fg	0.32	1.037818	0.32	0.784687	0.99	1.800537
10 fg	0.91	1.306484	-1.14	3.67389	-0.57	2.217942
1 fg	1.01	0.872028	1.57	2.725093	0.53	0.912213
NC	0		0			
t-test[d]	5.55		0.41		-0.54	
overall average	0.58[e]	0.5542[f]	0.13	1.1536	-0.13	0.9409
Average down to 1 pg	0.45	0.1658	0.03	0.2229	-0.46	0.4139

[(a)] Target DNA of S. Enteritidis *ATCC13076 or E. coli O157:H7 with and without 10 fg of IAC DNA.*

[(b)] Subtraction of Ct-value of target DNA obtained with IAC and Ct-value without IAC DNA, and its standard deviation (S.D.) [(c)]

[(d)] T-test (with a critical t-value of 1.94 with 6 degrees of freedom) from dilution series of target DNA per reaction

[(e)] Overall average of subtracted Ct-values and its corresponding S.D [(f)].

However, down to 1 pg of target DNA per reaction, the average of these differences (0.45 cycles) in combination with the average S.D. (± 0.17 cycles) revealed a more or less constant influence of IAC_E amplification on *S.* Enteritidis DNA amplification (increase of Ct-value with 0.45 ± 0.17 cycles).

In case of *E. coli* O157:H7 detection (*stx1*-gene and *stx2*-gene; $Ecol2_{Taq\text{-}sse}$), the t-test for paired data showed that the Ct-values of the dilution series of target DNA with and without co-amplification of the IAC_S DNA (1 pg of IAC_S DNA / PCR in case of both *E. coli* O157:H7 *stx1*-gene and *stx2*-gene detection) were similar (t-value *stx1*-gene: 0.41, t-value *stx2*-gene: 0.54, critical t-value: 1.94, when α=0.05 and 6 degrees of freedom; Table 2). This indicated that IAC co-amplification had no influence on the *E. coli* O157:H7 *stx1*-gene and *stx2*-gene amplification. A t-test for the *E. coli* O157:H7 *eae*-gene was not valid due to the overall high

variance (Figure 2).

Quantification of target DNA with co-amplification of the internal amplification control

The method $Salm_{Taq\text{-}invA}$ including the most optimal amount of IAC (10 fg of IAC_E DNA / PCR reaction) was further evaluated to determine the method boundaries with respect to precision and accuracy during target quantification. The mean Ct-value and S.D. (with n=12 and a 95% confidence interval for the mean) were calculated for each amount of target DNA tested and plotted versus the log [amount of target DNA] (Figure 5). All samples tested (n=96) were positive for the IAC (not shown). Amounts of 1 pg of *S.* Enteritidis DNA or higher showed an acceptable S.D. (<0.35 cycles), i.e. precision. Decreasing amounts of target DNA lower than 1 pg showed an increase in S.D. (>0.9 cycles). The method was accurate (R^2-value of 0.9928) in case target DNA amounts of 1 pg or higher were used.

Quantification based on Ct-values was not influenced by co-amplification of the IAC with respect to sample precision and was statistically precise down to amounts of 1 pg of target DNA (Figure 5).

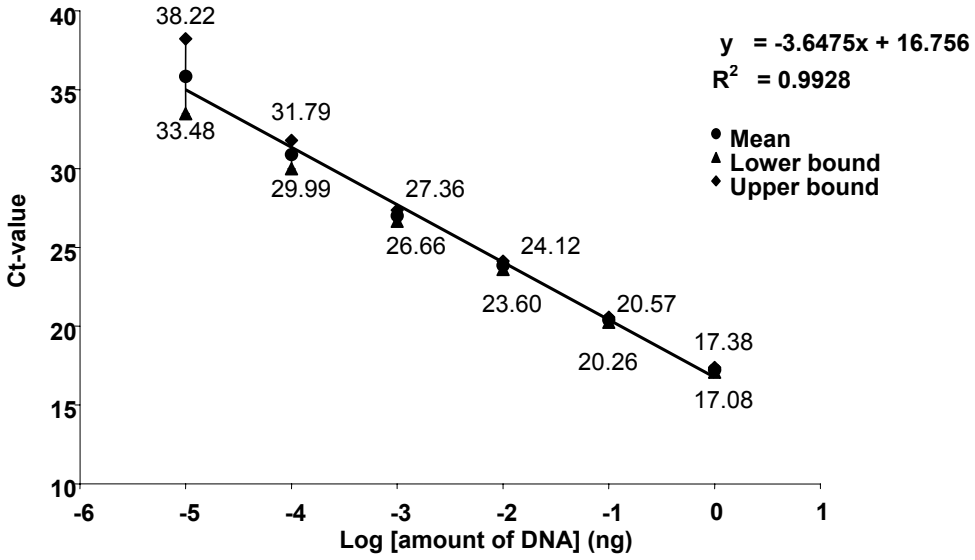


Figure 5. *The mean Ct-value and S.D. (of 12 repetitions) within each amount of DNA plotted versus the log [amount of DNA]. The lower and upper bound Ct-values (with a 95% confidence interval for the mean) with each amount of DNA tested are presented with each data point. The equation of the regression line and its R^2-value are displayed in the graph.*

Using the regression formula from figure 5 the initial amount of target DNA could be calculated as follows:

$$x = 10^{\left(\frac{(Ct-value-16.756)}{-3.6475}\right)} ng$$

,where x equals the initial amount of target DNA. According to calculations based on genome size, 1 ng of *S. enterica* target DNA is equal to 2x10^5 CFU *S. enterica*.

A maximum error of 0.34 x amount of target DNA (34 %) was obtained when testing amounts of 1 pg of target DNA (200 CFU ± 68 CFU (using the regression line formula, given in figure 5). An error of 0.07 x amount of target DNA (7%) was present in case 1 ng of target DNA amounts were tested (2 x 10^5 CFU ± 1.4 x 10^4 CFU). Quantification of amounts of initial target DNA lower than 1 pg was not possible due to high S.D., leading to only qualitative analysis within the region close to the detection limit.

Discussion

Until now, many real-time PCR methods have been described evaluating their sensitivity and specificity (for example Chen *et al.*, 2000; Fortin *et al.*, 2001; Kurowski *et al.*, 2002; Sharma *et al.*, 1999). However, only few papers describe the precision and accuracy of the methods evaluated (Jebbink *et al*., 2003; Stöcher and Berg, 2002). Even less papers are available presenting comparative studies of different real-time PCR methods for the detection of a specific organism (Jebbink *et al*., 2003), and none for detection of *S. enterica*, or *E.coli* O157:H7.

This study compared previously described real-time PCR methods for the detection of *S. enterica* and for the detection of *E. coli* O157:H7 with respect to sensitivity, precision and accuracy. A detection limit of 1 fg of *S.* Enteritidis DNA or *E. coli* O157:H7 DNA was obtained for qualitative analysis. A high precision and accuracy, needed for quantification, was obtained using amounts of at least 100 fg *S.* Enteritidis DNA or 100 fg *E. coli* O157:H7 DNA. The precision and accuracy were decreased close to the detection limit using the tested PCR methods, implying a high risk of errors for quantification in this region. Therefore, target amounts down to 2 log (or less) above the detection limit should only be estimated at a qualitative level. Quantitative analysis in this region will give rise to misinterpretation of the

data.

The most sensitive, precise and accurate real-time PCR methods were all Taqman-based methods rather than molecular beacon-based methods. Nevertheless, the sensitivity, precision and accuracy of a PCR-based method are not defined by the choice of detection probe (Taqman probe or molecular beacons), but mainly by the PCR performance itself (which is influenced by the primer sequence, primer specificity, annealing temperature, etc.). In line with this, Jebbink *et al*. (2003) found no difference in accuracy and reliability between real-time PCR assays using Taqman probes and molecular beacons for quantitative analysis of *Epstein-Barr virus* and *Cytomegalovirus*. Thus, using molecular beacons or Taqman probes for real-time PCR detection will not affect the sensitivity, precision or accuracy of the method.

The most sensitive and accurate methods (*S. enterica*: $Salm_{Taq\text{-}invA}$; *E. coli* O157:H7: $Ecol2_{Taq\text{-}sse}$) were improved by introduction of a general internal amplification control (IAC). The combination of the IAC with the real-time PCR methods to detect *S. enterica* or *E. coli* O157:H7 was evaluated, revealing that the precision and accuracy were retained, and the sensitivity was reduced only 10-fold. Still, using an internal amplification control with real-time PCR detection is important to identify false negative results and to control for presence of amplification inhibitors, especially for certified routine diagnostic laboratories (Hoorfar *et al*., 2000; Raggam *et al*., 2002).

Each previously described method for introduction of an IAC is limited either due to primer competition, or requires the presence of a specific substrate or organism, like in case house-keeping genes are used for gene-expression studies (Savli *et al*., 2003; Vandesompele *et al*., 2002). The new approach presented in this paper comprises a dual amplification of target DNA and IAC-DNA using a specific target primer set and IAC primer set. The latter is based on the *gfp*-coding sequence, which is derived from an organism not found in natural environmental substrates. Due to these features, the IAC-approach described in this paper is particularly suitable as a general IAC, which can be used irrespective of the substrate or target organism to be detected. But, due to the co-amplification of the IAC, a reduction in target sensitivity was observed. This is not surprising, since such reduction is inherent to the simultaneous amplification of different targets in one reaction (reagent competition). Nevertheless, only a minor influence on target sensitivity was introduced by co-amplification of the IAC, providing an acceptable and convenient tool for amplification control.

Although false positive results were not expected, an increase in fluorescence was observed when testing the $Ecol2_{Taq\text{-}rfbE}$ method with *S.* Typhimurium-*gfp* DNA. It might have been

introduced due to contamination problems or the presence of amplification artifacts obtained during PCR. In this particular case, repeated tests rejected to possibility of contamination. Therefore, the increase of fluorescence at the final cycles of PCR can be explained by the presence of PCR artifacts (like probe instability). Such artifacts are occasionally found with specific multiplex amplification tests, herewith reducing the applicability of the method. A redesign of the target probe often eliminates this problem. This might have prevented the false positive results observed from the method $Ecol_{MB\text{-}rfbE}$, but this was not tested any further in this study.

The co-amplification of the IAC did not affect the precision and accuracy of the target detection methods, allowing determination of the quantification threshold and qualitative detection limit of the $Salm_{Taq\text{-}invA}$ method for accurate initial target quantification. As co-amplification of the IAC did not affect quantification and the precision of the method was very high, initial amounts of target DNA could be calculated using the formula presented in this paper. This formula can be applied for quantification of *S. enterica* when using the Ct-values obtained from analysis of samples tested with $Salm_{Taq\text{-}invA}$ without the need for additional calibration curves within each experiment. Using this approach the Ct-value of the IAC may not differ from the expected IAC Ct-value (Ct-value of 31.5 cycles) to make sure no amplification inhibiting agents were present affecting target quantification. Of course, for external sample control a dilution series of *S. enterica* DNA including a fixed amount of IAC (10 fg DNA / reaction) might be used with each experiment for additional calibration. The subsequent regression plot obtained from this series can be used next to the formula described above for quantification of samples harboring unknown amounts *of S. enterica*.

The optimised methods for detection of *S. enterica* and *E.coli* O157:H7 including the IAC described in this study are useful tools for analysis of target DNA. However, in this paper only DNA extracted from pure cultures was tested. Detection of *S. enterica* or *E.coli* O157:H7 in substrates like manure, meat or poultry might introduce a reduction in sensitivity, precision or accuracy. Investigations to study the influence of environmental substrates on detection of *S. enterica* with respect to sensitivity, precision and accuracy showed promising results, especially with respect to the applicability of the IAC to control DNA extraction and target amplification. Such control might be more favourable over other approaches, since it controls the whole procedure of DNA extraction and amplification until detection.

In conclusion, the optimised real-time PCR detection for either *S. enterica* or *E. coli* O157:H7 may provide an improved, sensitive, precise and accurate method, applicable for high-throughput analysis and routine diagnosis, preventing false negative reactions, providing a

tool for accurate quantification of *S. enterica* or *E. coli* O157:H7.

Acknowledgements

This research was supported by the Produktschap Tuinbouw, The Netherlands, and the Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs. The authors would like to thank Ir. C. Warmerdam and Dr. H. Aarts from RIKILT, The Netherlands for providing *S.* Enteritidis ATCC 13076, Dr. F. van Leusden from RIVM, The Netherlands for providing *E. coli* O157:H7, strain 102, Dr. Römling from Karolinska Institutet, Microbiology and Tumorbiology Center, Sweden for providing *gfp*-modified *S.* Typhimurium strain 110, and Dr. Fratamico from Agricultural Research Service, Eastern Regional Research Center, U.S. Department of Agriculture, USA for providing *gfp*-modified *E.coli* O157:H7. The authors would like to thank Dr. A.M. Semenov for constructive discussions and for reviewing the manuscript.

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Chapter 3

Comparison of methods of extracting *Salmonella enterica* serovar Enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control

Applied and Environmental Microbiology (2006) **72:**3879-3886

Comparison of methods of extracting *Salmonella enterica* serovar Enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control

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Summary

This paper compares five commercially available DNA extraction methods with respect to DNA extraction efficiency of *Salmonella enterica* serovar Enteritidis from soil, manure and compost and uses an *Escherichia coli* strain harboring a plasmid expressing Green Fluorescent Protein as a general internal procedural control. Inclusion of this general internal procedural control permitted more accurate quantification of extraction and amplification of *S.* Enteritidis in these samples and reduced the possibility of false-negatives. With this protocol it was found that the optimal extraction method differed for soil (Mobio soil DNA extraction kit), manure (Bio101 soil DNA extraction kit) and compost (Mobio fecal DNA extraction kit). With each method, as little as 1.2-1.8 x 10E3 CFU of added S. Enteritidis/100 mg substrate could be detected by direct DNA extraction and subsequent *S. enterica* specific Taqman PCR. After bacterial enrichment, as little as 1 CFU/100 mg of original substrate was detected. Finally, the study presents a more accurate molecular analysis for quantification of *S.* Enteritidis initially present in soil or manure using DNA extraction and Taqman PCR.

Introduction

Environmental substrates like manure and soil have become a major concern with respect to food safety, since these substrates are suspected to play a major role in the introduction of human pathogens in the food chain (Natvig *et al.*, 2002; Solomon *et al.*, 2002; Wang and Doyle, 1998). For example, *Salmonellae* are frequently found in association with animal manure (Franz *et al.*, 2005; Lettelier, 1999; Natvig *et al.*, 2002; Pell, 1997) which is often applied as fertilizer to soil prior to vegetable production, herewith introducing a potential risk of contamination of vegetables grown in the manure-amended soil. This threat is evident from the fact that during recent years the consumption of raw vegetables has been related to food-borne outbreaks (Anonymous, 1999; Behrsing *et al.*, 2000; Fisher, 2004; Hilborn *et al.*, 1999; Lyytikainen *et al.*, 2004; Michino *et al.*, 1999; Robertson *et al.*, 2002). For example, each year 3.5 million cases of *Salmonellosis* occur in the US and Canada that lead to economic losses up to 3.4 billion $ a year (Todd, 1989). Therefore, the detection and quantification of pathogens like *S. enterica* that are present in environmental substrates like soil, manure and compost, is of high importance. It will enable risk assessment and pathogen monitoring at different stages in the plant production chain and ensure food health and safety in the food industry.

Standardized diagnostic procedures to detect the presence of *S. enterica* in food samples (ISO 6579:2002) are mainly based on microbiological culturing methods, which in general require up to five days until results are obtained (Stewart *et al.*, 1998). In order to reduce the time demand, alternative techniques like immunological assays (Acheson *et al.*, 1994; Bolton *et al.*, 2000) and molecular methods (Bej *et al.*, 1994; De Medici *et al.*, 2003; Heller *et al.*, 2003) have been applied to detect *S. enterica* in various samples. Especially real-time PCR methods such as 5' nuclease Taqman PCR (Holland *et al.*, 1991) have shown promising results due to the rapid, sensitive and specific detection of *S. enterica* (Bassler *et al.*, 1995; Higgins *et al.*, 1998; Hoorfar *et al.*, 2000; Klerks *et al.*, 2004).

However, molecular methods like (Taqman) PCR are limited by the fact that these are dependent on the suitability of the extracted DNA for PCR (Wilson, 1997). Especially DNA extracted from soil, manure or compost can have co-extracted contaminants like humic and fulvic acids known to cause problems during PCR amplification (Al-Soud and Rådström, 1998; Fortin *et al.*, 2004). Other components different from humic and fulvic acids but commonly present in soil, have also been related to PCR inhibition (Watson and Blackwell, 2000). Moreover, the large variation in biochemical components between different substrates

(Al-Soud and Rådström, 1998; Wilson, 1997) usually leads to variable efficiencies of DNA extraction methods (Lloyd-Jones and Hunter, 2001; Theron and Cloete, 2000). Due to these deficiencies, accurate quantification of pathogens present in different environmental substrates has not yet been accomplished using molecular techniques such as PCR.

To control the effects of inhibiting agents on PCR amplification efficiency, Taqman PCR was improved recently by introduction of a general internal amplification control to prevent the occurrence of false negative results (Hoorfar *et al.*, 2000; Klerks *et al.*, 2004; Raggam *et al.*, 2002). Although this improvement provided progress in the analysis of extracted DNA from environmental substrates, a comparison between the DNA extraction efficiency of different DNA extraction methods has only been described to a minor extent. Zhou *et al*. (1996) investigated the DNA recovery from different soils, but only one DNA extraction method was used. Another paper described a comparison of three different DNA extraction methods, evaluating the DNA quality and quantity recovered from four soils with widely differing characteristics, but not from manure or compost (Lloyd-Jones and Hunter, 2001).

The objectives of this study were to evaluate five commercial DNA extraction methods with respect to DNA extraction efficiency from soil, manure and compost. In addition, the development and application of a general internal procedural control was investigated with respect to the efficiency of the DNA extraction and Taqman PCR amplification procedure. Moreover, the possibility of a more accurate quantification of *Salmonella enterica* serovar Enteritidis from different substrates by using the general internal procedural control was evaluated.

Materials and Methods

Bacterial strains and environmental substrates

A liquid culture of *Salmonella enterica* serovar Enteritidis ATCC 13076, grown overnight at 30°C in tryptic soy broth, was kindly provided by Dr. H. Aarts (RIKILT, Wageningen, The Netherlands). A bacterial culture of the genetically modified *Escherichia coli* strain 99507*gfp*, containing a plasmid pVSP61TIR (Miller and Lindow, 1997) expressing green fluorescent protein (*gfp*) (Prasher *et al*., 1992), was kindly provided on solid LB medium by Dr. R. Sayler (Semenov *et al.*, 2004). Each *E.coli* strain 99507*gfp* colony forming unit contains approximately 30 plasmids with a coding sequence for gfp expression. A subculture of *E. coli* strain 99507*gfp* was grown overnight at 37°C in liquid broth (LB) medium containing 50 µg/ml

ampicillin. The bacterial suspension was diluted in 50% glycerol, divided into aliquots and stored at –80°C for further use.

Soil samples S4O, S4C, S5O, S5C, S7O, S7C, S9O and S9C, manure samples M1, M2, M3, M4, M5 and M6, and compost samples CA and CB were obtained from Dr. A. van Diepeningen (Wageningen University and Research Centre, Biological Farming Systems, Wageningen, The Netherlands) who characterized and described the soil and manure samples extensively (Franz *et al.*, 2005; Van Diepeningen *et al.*, 2005). The selection of environmental samples was primarily based on a wide difference in microbial community and the presence of compounds that are suspected to inhibit or influence DNA extraction and/or PCR amplification. This was taken into account in order to cover the most common problems when dealing with DNA extraction and amplification from environmental samples. Soils S4 and S9 were sandy soils with 3 to 3.5% clay, 10-33% silt and 64-87% sand. Soils S5 and S7 were loamy soils with 8-11% clay, 40-55% silt, and 37-51% sand. Each soil was represented by two composite samples from neighboring organic and conventional farms. Manures were collected from individual Friesian Holstein cows with different diets (Franz *et al.*, 2005). Composts originated from green garden waste (CA) and green household waste (CB), and were obtained from two large composting facilities. Each type of substrate used in this study tested negative for naturally present *S. enterica* in a test with bacterial enrichment of each substrate type, followed by both plating on selective Hektoen enteric agar, and DNA extraction and subsequent *S. enterica*-specific Taqman PCR detection.

DNA extraction

The following commercial DNA extraction methods were used: Ultraclean Soil DNA isolation kit ($Mobio_{soil}$) (MoBio Laboratories, Solana Beach, USA), Ultraclean Fecal DNA kit ($Mobio_{fecal}$) (MoBio), Bio101 extraction kit (Bio101) (Q-Biogene, Carlsbad, USA), Soilmaster DNA extraction kit (Epi_{soil}) (Epicentre, Madison, USA), Plant DNeasy DNA extraction kit (Qia_{dneasy}) (Qiagen, Westburg), and a combination of the Microbial DNA extraction kit ($Mobio_{micro}$) (MoBio) with bacterial isolation using Optiprep (60% w/v solution of iodixanol; Axis-Shield, Oslo, Norway) at a density of 1.320 g/ml. All DNA extraction methods were performed following the manufacturers' instructions, including bead beating of the samples on a flatbed shaker at 250 rpm. To separate bacteria from soil using Optiprep, 400 µl of buffered peptone water (BPW) was added to 100 mg of soil sample and mixed by vortexing. Subsequently, a layer of 250 µl of Optiprep was pipetted underneath the soil suspension. The tubes were centrifuged at maximum speed (14000 rpm) for 5 min. All supernatant on top of the Optiprep and the Optiprep solution itself was transferred to a clean tube. The suspension was mixed with 750 µl BPW and centrifuged again at maximum speed for 5 min. The supernatant was discarded prior to further DNA extraction with the $Mobio_{micro}$ method.

Primers and probes

Sequences of the primers and probe for detection of *S. enterica* using Taqman PCR were derived from Hoorfar *et al*. (2000). To reduce false negative results and provide a more accurate quantification of *S.* Enteritidis in substrate samples, a general internal procedural control (GIPC) was used. Detection of the GIPC was based on a *gfp*-gene present in an *E.coli* strain harboring a multicopy plasmid containing the *gfp*-gene. For detection of the *gfp*-gene of the internal extraction and amplification control using Taqman PCR, the sequences of the primers and probe were obtained from Klerks *et al*. (2004). To allow the simultaneous detection of both targets, the *S. enterica* specific detection probe was labeled at the 5' end with 6-carboxyfluorescein (FAM), whereas the *gfp*-gene specific probe was labeled at the 5' end with Yakima Yellow (Eurogentec, Maastricht, The Netherlands). Both detection probes were labeled at the 3' end with Eclipse Dark Quencher (Eurogentec).

Preparation of bacterial dilution series and plate counting

A fresh liquid culture of *S.* Enteritidis ATCC 13076 was maintained by daily picking two colonies from selective Hektoen enteric agar (Biotec Laboratories Ltd.,UK) and growing the colonies separately in BPW overnight at 37°C and 250 rpm. A dilution series of *S.* Enteritidis was prepared each time by diluting fresh liquid culture 10-fold, up to a 10^8 times dilution (9 different dilutions in total, and 1 negative control). To determine the amount of *S.* Enteritidis CFU present in each dilution series, 40 µl of each dilution was plated on selective XLD (xylose lysine de-oxycholate) agar (Biotec Laboratories Ltd.,UK) and selective Hektoen enteric agar in duplicate. The selective plates were incubated overnight at 37°C and the number of colonies was determined for each plate.

Development of an internal extraction and amplification control

As a procedural control, whole cells of *E.coli* strain 99507*gfp* were added to a substrate sample prior to DNA extraction and amplification. First, to determine the optimal amount of *E.coli* strain 99507*gfp* to be added to a sample prior to extraction, a 10-fold dilution series (up to 10^8 times diluted) of a liquid culture of *E.coli* strain 99507*gfp* (previously stored at –80°C in 50% glycerol) was prepared in BPW. Ten µl of the 10-fold dilution series and a negative control were added to soil S4O (100 mg of soil per sample) prior to DNA extraction using the $Mobio_{soil}$ method. The extracted DNA was diluted 10-fold before analysis by *gfp*-specific Taqman PCR. The most optimal amount of *E.coli* strain 99507*gfp* was defined by that dilution factor resulting in a Ct-value close to 31.5 (Klerks *et al.*, 2004). The Ct-value is defined by the number of cycles resulting in a detectable fluorescence signal above the threshold, defined by the mean plus four times the S.D. of the fluorescence signal of the

control samples.

The 10-fold dilution series (40 µl of each dilution) was also plated onto LB-agar containing 50 µg/ml ampicillin and incubated at 37°C overnight. The number of colonies was counted on each plate and the number of *S.* Enteritidis CFU's was calculated for each dilution of the dilution series added prior to DNA extraction.

Real-time PCR and internal control amplification

The improved real-time Taqman PCR method to simultaneously detect *S. enterica* and an internal amplification control (IAC) (Klerks *et al.*, 2004) was used throughout all experiments. Prior to DNA extraction, *E.coli* strain 99507*gfp* ($2.5x10^4$ CFU) was added to each substrate sample. Subsequent to DNA extraction, 2.5 µl of a ten-fold diluted extracted DNA sample was used for PCR amplification (PCR total volume of 30 µl), as previously described (Klerks et al., 2004). Taqman PCR was performed using the qPCR core kit (Eurogentec) and amplification was measured using the ABI Prism 7700 (Perkin Elmer, Norwalk, CT). Each DNA sample was tested by Taqman PCR in triplicate.

Comparison of different DNA extraction methods with respect to extraction efficiency

To determine the most favorable method to use for DNA extraction from soil, manure or compost, the different methods were compared with respect to extraction efficiency. The extraction methods $Mobio_{soil}$, Epi_{soil}, Qia_{dneasy}, Bio101 and $Mobio_{micro}$ (with prior Optiprep) were compared using a subset of soils (soil S4O, S5O and S9O), since most extraction difficulties were expected for organically managed soils. The methods $Mobio_{soil}$, $Mobio_{fecal}$, Epi_{soil}, Qia_{dneasy} and Bio101 were compared using a subset of manures (M1, M2 and M3) and one compost (CA). The DNA extraction efficiency was defined by the Ct-values obtained with Taqman PCR, which indicated the suitability of the extracted DNA for PCR amplification.

First, 10 µl of a dilution series (non-diluted, 10, 100 and 1000 times diluted) of *S.* Enteritidis liquid culture was added to 100 mg of soil S4O, S5O, S9O, manure M1, M2, M3 and compost CA, in duplicate. Subsequently, DNA was extracted using the different DNA extraction methods and diluted 10-fold prior to downstream analyses.

Finally, each diluted DNA sample was subjected to Taqman PCR including an internal amplification control (IAC: 10 fg of *E. coli* strain 99507*gfp* DNA; Klerks *et al.*, 2004). The simultaneous *S. enterica* amplification and IAC co-amplification (in one tube) was followed in real-time using the Abi Prism 7700.

Consistency of the internal procedural control with different substrates

To evaluate whether the previously (section above) determined optimal amount of *E.coli* strain 99507*gfp* (2.5 x 10^4 CFU, resulting in a Ct-value of 31.5) showed consistent results in Taqman PCR, it was tested with all soils, manures and composts present. From each substrate 100 mg was used for sample preparation and DNA extraction. In each sample of soil, manure and compost, 2.5 x 10^4 CFU *E.coli* strain 99507*gfp* was added and DNA was extracted using the $Mobio_{soil}$, the Bio101 and the $Mobio_{fecal}$ method, respectively, for soil, manure and compost samples. After DNA extraction all purified DNA samples were diluted 10-fold and 2.5 µl of each diluted sample was tested using the *gfp*-specific Taqman PCR.

Evaluation of quantitative detection of Salmonella enterica extracted from soil, manure or compost

First, the precision of DNA extraction and the extraction efficiency (defined by the recovery of added *S.* Enteritidis based on the Ct-values obtained from PCR) of the most optimal extraction methods (soil, $Mobio_{soil}$; manure, Bio101; compost, $Mobio_{fecal}$) was tested in a large-scale evaluation. To accomplish this, the substrates were divided into separate groups, since DNA extraction and further analysis from only one group per day appeared feasible. Group 1 consisted of soil S4O, S4C, soil S5O, S5C; group 2 of soil S7O, S7C, S9O, S9C; group 3 of manure M1, M2, manure M3, M4; and group 4 of manure M5, M6, compost CA, CB. Each group was treated in a similar manner using fresh bacterial cultures each day.

A 10-fold dilution series of *S.* Enteritidis was prepared. In duplicate, 40µl of each of the five largest dilutions (10^5 times / 10^6 times / 10^7 times / 10^8 times / negative control) was plated on selective XLD agar and Hektoen enteric agar and incubated at 37°C overnight prior to colony counting. In addition to plating, 10 µl of each dilution was added to 10 ml tubes containing 100 mg of substrate. BPW (2 ml) was added to each tube and incubated overnight at 37°C and 250 rpm. To extract DNA from enrichment samples, 1 ml of the enrichment culture was transferred to a clean tube and 10 µl of GIPC (2.5x10^6 CFU *E.coli* strain 99507*gfp* / ml) was added. DNA was extracted using the $Mobio_{micro}$ DNA extraction method, diluted 10-fold and stored at –20°C.

Then, 10 µl of each *S.* Enteritidis dilution was added to 100 mg of each substrate, followed by the addition of 10 µl of 2.5x10^6 CFU / ml GIPC to each substrate sample. DNA was extracted from the soil, manure and compost samples using, respectively, the $Mobio_{soil}$, the Bio101 and the $Mobio_{fecal}$ method. The purified DNA was diluted 10-fold and stored at –20°C. All stored DNA samples were finally analysed by performing Taqman PCR to detect *S.* Enteritidis and the GIPC simultaneously, in triplicate.

Statistical analysis

The most efficient method to use for DNA extraction per soil, manure or compost was determined based on the extraction efficiency, i.e. the Ct-value (the number of cycles resulting in a detectable fluorescence signal above the threshold, defined by the mean plus four times the S.D. of the fluorescence signal of the control samples) obtained from Taqman PCR. The methods were compared by performing a univariate analysis of variance (ANOVA). The mean Ct-values were calculated from the different substrates per substrate type and per dilution factor, and a Post hoc Tukey's test with a 95% mean confidence interval was performed.

The applicability of using a set number ($2.5x10^4$ CFU) of whole cells of *E.coli* strain 99507*gfp* (GIPC) to control DNA extraction and amplification was determined per substrate (eight soils, six manures and two composts, in triplicate) by calculating the mean Ct-value and its corresponding precision, i.e. the S.D. The GIPC was considered applicable if the obtained mean Ct-value was 31.5 +/-1 Ct.

The selected methods were evaluated for their precision and extraction efficiency of GIPC DNA from different soils, manures or composts that had been amended with a dilution series of *S.* Enteritidis and the GIPC. ANOVA was performed on the Ct-values of the GIPC from the samples that were not positive for *S.* Enteritidis when using Taqman PCR (n=5 / substrate, tested in triplicate with PCR). The mean Ct-values of the GIPC from the different substrates per substrate type were compared by performing a Post hoc Tukey's test. The extraction precision of each DNA extraction method was estimated as the coefficient of determination for linear regression of the mean Ct-values versus the log [number of *S.* Enteritidis CFU / 100 mg substrate] per substrate.

The effect of time of sampling (4 sampling days) was assessed by multivariate ANOVA including a post-hoc Tukey's test. As there were no significant differences among extraction dates for the different substrates, a regression line of all Ct values versus CFUs per 100 mg of soil, manure or compost was calculated for each substrate, including 95% confidence intervals.

Results

Comparison of methods to extract DNA from soil, manure or compost

The efficiency of DNA extraction from the different soils was most optimal if the $Mobio_{micro}$ method was used (mean Ct-value of 28.8; Table 1). Except for the $Mobio_{micro}$ method, the DNA extraction efficiency of each of the 5 methods was very low for soil S9O (high Ct values, Table 1). After omitting this soil from the statistical analysis, the $Mobio_{micro}$ method was not significantly different from the $Mobio_{soil}$ method (p=0.962) and the Epi_{soil} (p= 0.087), but the latter two methods were significantly different from each other (p= 0.016). Omitting soil S9O and the $Mobio_{micro}$ method from the analysis, the $Mobio_{soil}$ method was most efficient for subsequent PCR amplification (adjusted mean Ct-value of 28.4 in Table 1).

Table 1. *Mean Ct-values of Taqman PCR on DNA from a dilution series of* Salmonella *Enteritidis added to 100 mg of soil, manure or compost prior to DNA extraction, and univariate analysis of variance of DNA extraction efficiency with Post-hoc Tukey's test between DNA extraction methods.*

Substrate	Sample	$Mobio_{fecal}$	$Mobio_{soil}$	Epi_{soil}	Qia_{dneasy}	Bio101	$Mobio_{micro}$
	S4O		28.69[I]	31.11	40.00	32.70	29.21
	S5O		28.12	30.80	34.90	33.95	28.65
	S9O		38.75	35.57	40.00	40.00	28.56
Soil							
	Mean[II]		31.85 *b*[III]	32.49 *b*	38.30 *d*	35.55 *c*	28.81 *a*
	Adjusted mean[IV]		28.40 *a*	30.95 *b*	37.45 *d*	33.33 *c*	
Manure	M1	28.64	29.05	35.87	26.18	25.10	
	M2	30.16	38.19	36.73	31.60	25.49	
	M3	28.86	27.78	35.09	25.69	26.15	
	mean	29.22 *c*	31.67 *d*	35.89 *e*	27.82 *b*	25.58 *a*	
Compost	CA	28.63 *a*	29.80 *a*	36.33 *b*	40.00 *c*	40.00 *c*	

[I] *Displays the mean Ct-value obtained per substrate*

[II] *The mean Ct-value of the substrate obtained with each DNA extraction method*

[III] *Displays the homogeneous subsets obtained from the Post-hoc Tukey's test, using an harmonic mean sample size = 24 and alpha=0.05, separating the methods from high to low (from a to e) DNA extraction efficiency.*

[IV] *Presents the adjusted mean, based on Post-hoc Tukey's test performed without the data from soil 9O and without the $Mobio_{micro}$ method, with a harmonic mean sample size = 16 and alpha = 0.05.*

The Bio_{101} method (mean Ct-value of 25.58) resulted in the most optimal DNA extraction efficiency for each manure. Each method tested appeared significantly different with respect to DNA extraction efficiency, irrespective of the manure tested (Table 1).

General internal procedural control for DNA extraction and amplification

When 5 x 10^4 CFU or 5 x 10^3 CFU of general internal procedural control (GIPC; *E.coli* strain 99507*gfp*) was added to 100mg of substrate prior to DNA extraction, PCR amplification resulted in a Ct-value of 30 or 33, respectively (Figure 1). The optimal amount resulting in a Ct-value of 31.5 (19) was obtained by adding 2.5 x 10^4 CFU of GIPC to 100 mg of each substrate prior to DNA extraction. The Ct values for the GIPC varied very little, when 2.5 x 10^4 CFU of GIPC was added to 100 mg of the various substrates, ranging from 31.32 $\pm$ 0.33 (mean Ct $\pm$ SD) for soil extracted with $Mobio_{soil}$, 30.72 $\pm$ 0.83 for manure extracted with Bio101, to 32.38 $\pm$ 0.26 for compost extracted with $Mobio_{fecal}$.

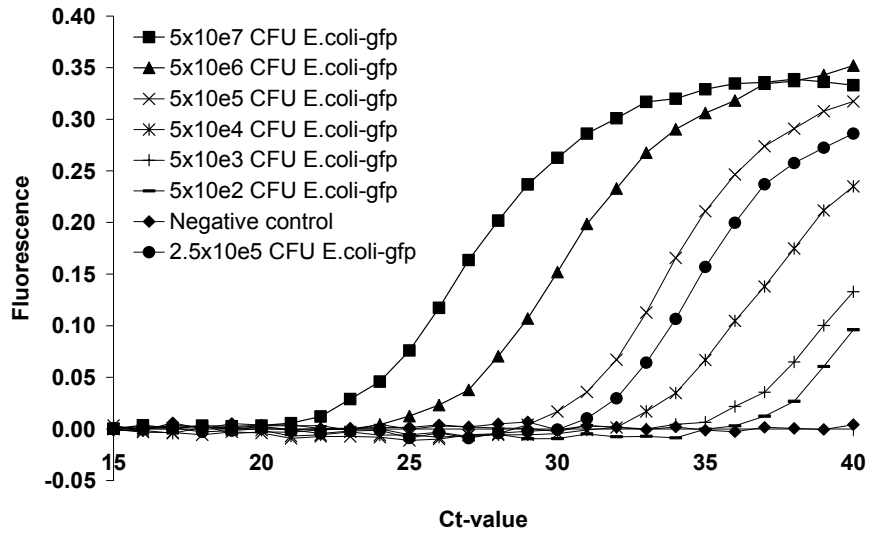


Figure 1. *Real-time amplification and detection of a ten-fold dilution series of* E. coli *strain 99507gfp ranging from non-diluted bacterial culture to 10^5 times diluted bacterial culture added to soil prior to DNA extraction (equal to 5x10^6 CFU / DNA extraction down to 50 CFU / DNA extraction), including the optimal amount of* E.coli *strain 99507gfp (giving a Ct-value of 31). The fluorescence increase is plotted versus the cycle number of PCR.*

Evaluation of the selected S. enterica detection procedure from soil samples

DNA was extracted from 100 mg of soil (using $Mobio_{soil}$), including the GIPC (2.5×10^4 CFU / DNA extraction) and a dilution series of *S.* Enteritidis. GIPC DNA extracted from eight different soils was similar for all soils (p=0.056) except for S4O. The mean GIPC Ct-value of the subset of 7 soils was 31.48 ± 0.94, while that of S4O was 29.21 ± 0.93 (Table 2).

S. Enteritidis extracted directly from 100 mg of soil was detected by Taqman PCR at a range from 1.6×10^7 down to 1.6×10^3 CFU / 100 mg soil (equal to 7.8 CFU / PCR) (Table 3). After enrichment of the soil samples inoculated with *S.* Enteritidis, *S.* Enteritidis was detected even when only 1 CFU (calculated amount) was originally added to 100 mg of soil (Table 3).

Evaluation of the selected S. enterica detection procedure from manure samples

The amount of GIPC DNA extracted from 6 manures using Bio101 was quite consistent, for a sub-set of those manures (M1, M3, M4 and M6) with a mean Ct-value of 28.98 and a SD of 0.62, (p=0.805). The mean Ct values of this subset differed significantly (p=0.001-0.026) from those of another subset (M2 and M5) with mean Ct values of 31.64 ± 1.08 and 33.85 ± 2.05, respectively (Table 2).

S. Enteritidis was detected by Taqman PCR at a range from 1.8×10^7 down to 1.8×10^3 CFU / 100 mg manure (equal to 9.2 CFU / PCR) when extracted directly from manure (Table 3). Detection of *Salmonella* DNA extracted from manure M2 was not possible. Manure M5 was only positive for *S.* Enteritidis when high amounts (10^6-10^7 CFU) of *S.* Enteritidis were added to 100 mg of substrate prior to DNA extraction (Table 3). Enrichment of manures inoculated with *S.* Enteritidis enabled the detection of even 1 CFU of *S.* Enteritidis originally added per 100 mg of each manure (Table 3).

Evaluation of the selected S. enterica detection procedure from compost samples

GIPC DNA extraction from compost with $Mobio_{fecal}$ resulted in significantly different (p=0.03) Ct-values for compost CA and CB, with mean Ct-values of 32.88 ± 1.84 and 31.41 ± 0.78, respectively. *S.* Enteritidis extracted from compost CB was detected by Taqman PCR in a range of 1.2×10^7 CFU down to 1.2×10^3 CFU / 100 mg compost (equal to 6.2 CFU / PCR) (Table 2). DNA extraction from compost CA was less efficient than that from CB (Table 3). Enrichment of *S.* Enteritidis inoculated in the compost samples enabled the detection of even 1 CFU originally added to 100 mg of both compost samples (Table 3).

Table 2. *Mean Ct-values of Taqman PCR for the general internal procedural control (GIPC) added at a concentration of 2.5x10^4 CFU / 100 mg substrate prior to DNA extraction, and analysis of variance with post-hoc Tukey's test between substrates. DNA from the soil samples was extracted using the Mobio$_{soil}$ method, and DNA from the manure samples with the Bio$_{101}$ method.*

Substrate Soil	N[(I)]	Mean Ct-value[II)]	S.D.[(III)]	Substrate Manure	N	Mean Ct-value	S.D.
S4O	5	29.21 *a*[IV]	0.93	M1	5	28.52 *a*	0.70
S4C	5	31.31 *b*	1.52	M2	5	31.64 *b*	1.08
S5O	5	30.87 *b*	0.79	M3	5	29.36 *a*	0.30
S5C	5	30.70 *b*	0.80	M4	5	29.02 *a*	0.53
S7O	5	31.47 *b*	0.14	M5	5	33.85 *c*	2.05
S7C	5	31.92 *b*	0.50	M6	5	29.01 *a*	0.73
S9O	5	32.36 *b*	1.03				
S9C	5	31.74 *b*	0.26				
Subset *b*		31.48	0.94	Subset *a*		28.98	0.63

[(I)] The total number of samples where no S. Enteritidis *(negative controls and samples below detection limit) was detected by Taqman PCR.*

[(II)] Mean Ct-value obtained from Taqman PCR for the general internal procedural control (GIPC).

[(III)] Standard deviation of (b).

[(IV)] Displays the significant sub-groups calculated from the Ct-values of the GIPC using Tukey's test.

Note: with soil S4O too much (10-fold) GIPC was added to the sample prior to DNA extraction, resulting in a Ct-value significantly lower than that from the other soils.

Quantification of S. Enteritidis *present in various environmental substrates*

Regression of *S. enterica* Ct-values on the log [amount of target CFU] for each soil, manure and compost separately, resulted in a good fit for the *Salmonella* dilution series tested (R^2 =0.90-0.99), except for manures M2 and M5 (no regression analysis possible) and compost CA (R^2-value of 0.87).

Slope and intercept of the regression lines for soil samples analysed on 4 subsequent days (groups 1 to 4) were not significantly different in a MANOVA test (p=0.863 and p= 0.624 for slope and intercept, respectively). These results were confirmed by a post-hoc Tukey's test on the slope (p=0.832) and intercept (p= 0.582) for each group. Similar slopes and intercepts were found when testing a dilution series of *S.* Enteritidis with three different soils S4O, S5C and S9O simultaneously in one day (data not shown).

Table 3. *Ct-values obtained from Taqman PCR on DNA extracted directly and after enrichment from substrates that were amended with a dilution series of* S. *Enteritidis. The Ct-values are displayed per amount of* S. *Enteritidis (calculated from plating)*

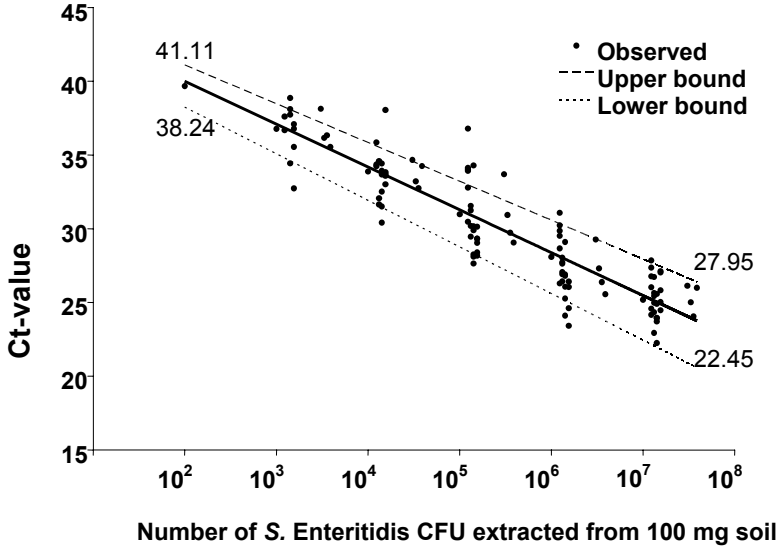
Method		**Soil**								**Manure**						**Compost**	
Direct	CFU																
	S. Enteritidis	S4O[b]	S4C	S5O	S5C	S7O	S7C	S9O	S9C	M1	M2	M3	M4	M5	M6	CA	CB
	Z x 10^{7}[a]	25.18	27.09	25.79	24.24	24.41	23.70	26.48	25.52	21.01	n[c]	20.20	20.05	23.04	21.13	n	24.39
	Z x 10^{6}	28.40	30.15	27.53	27.08	26.08	26.68	26.42	24.70	23.78	n	24.36	24.16	33.76	25.26	26.48	25.72
	Z x 10^{5}	32.88	34.50	30.30	31.56	30.61	28.85	28.63	29.51	27.57	n	27.27	27.40	n	28.43	32.69	29.54
	Z x 10^{4}	34.24	35.11	33.10	33.33	33.63	31.87	33.31	35.20	31.50	n	30.90	31.65	n	34.69	33.98	32.77
	Z x 10^{3}	37.15	39.12	36.17	n	38.24	34.44	36.17	34.92	36.40	n	36.52	34.83	n	n	n	37.37
	Z x 10^{2}	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	Neg.	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Enrichment																	
	Z x 10^{2}	17.38	15.73	15.74	16.16	17.47	15.75	16.06	15.86	16.93	15.62	16.80	16.96	16.09	16.15	20.71	24.63
	Z x 10^{1}	18.69	16.76	16.80	16.28	19.68	18.09	17.64	16.59	19.46	16.26	17.28	19.32	16.23	16.11	22.27	24.20
	Z x 10^{0}	20.90	18.63	17.58	17.17	21.51	34.51	19.39	18.38	22.16	15.98	34.81	21.19	16.20	16.99	23.88	33.31
	Z x 10^{-1}	n	n	n	n	n	n	n	19.84	22.42	n	n	n	n	n	n	n
	Neg.	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n

[a]*Number of calculated amount of colony forming units (CFU) of* Salmonella *Enteritidis added to 100 mg of each substrate prior to DNA extraction. Z was different for each time-related group (group 1, soil S4O, S4C: Z=1.2; group 2, soil S5O, S5C: Z=1.3; group 3, soil S7O, S7C:Z=1.4; group4, S9O, S9C: Z=1.6; group 5, manure M1, M2: Z=1.7; group 6, manure M3, M4: Z=0.8; group 7, manure M5, M6: Z=1.8; group 8, compost A, B: Z=1.2). Each group was treated in one day and in a similar manner using fresh bacterial cultures each time.*

[b]*Represents the substrate tested for DNA extraction and subsequent Taqman PCR.*

[c]*A sample negative for* S. Enteritidis *is indicated by (n).*

2A



2B

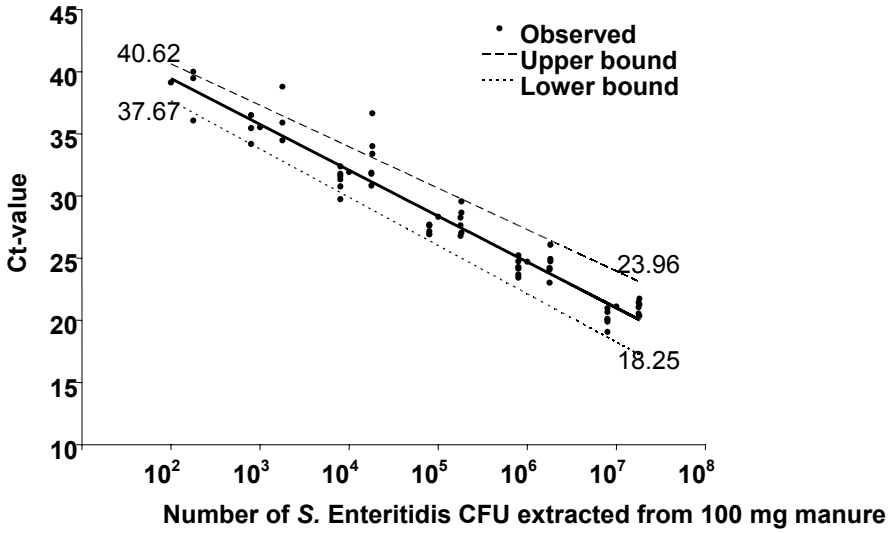


Figure 2. *Linear regression with 95% confidence intervals from ten-fold dilution series of* S. *Enteritidis added to soil (A) and manure (B) prior to DNA extraction and amplification. The Ct-value is plotted versus the log [number of* S. Enteritidis *CFU / 100 mg substrate].*

Subsequently, a regression line was estimated for all data per substrate (soil or manure), and 95% confidence intervals were calculated. The regression equations for soil (intercept = 42.571 +/- 1.17; slope = -2.896 +/- 0.264; R^2=0.841) and manure (intercept = 42.756 +/- 1.199; slope = -3.608 +/- 0.277; R^2=0.923) were not significantly different (Figure 2). The

variation in Ct values around the means (within the 95% confidence limits) ranged from 1.43 to 2.75 (equal to 2.70 to 6.73 times the difference in initial CFU / 100 mg soil) for the different concentrations of *S.* Enteritidis added to soil (Figure 2A). For manure, the variations in Ct values around the means were remarkably similar to those of soil, namely 1.48 to 2.86 (equal to 2.78 to 7.25 times the difference in initial CFU / 100 mg manure) for the different concentrations of *S.* Enteritidis added to manure (Figure 2B).

Discussion

Until now, many different DNA extraction methods have been described allowing subsequent PCR. However, only few papers describe a comparison of commercial DNA extraction methods with respect to extraction efficiency from soil, manure or compost. Zhou *et al*. (1996) described the development of a method to extract DNA from soil and evaluated the DNA recovery from eight soils with diverse composition using only this method. Lloyd-Jones and Hunter (2001) compared three different DNA extraction methods with respect to DNA recovery from four soils with different composition.

In this study six commercial DNA extraction methods were compared with respect to DNA extraction efficiency and quantification accuracy of *S.* Enteritidis initially present in the substrate sample. The Ultraclean Soil DNA isolation kit ($Mobio_{soil}$), the Bio101 extraction kit (Bio101) and the Ultraclean Fecal DNA kit ($Mobio_{fecal}$) were found most efficient for DNA extraction, from respectively soil (eight different substrates), manure (six substrates) and compost (two substrates). A sensitivity of approximately 10 CFU *S.* Enteritidis / PCR (2×10^3 CFU / 100mg substrate) was obtained using DNA extraction followed by *S. enterica* specific Taqman PCR. In addition, even 1 CFU of *S.* Enteritidis / 100 mg of substrate was clearly detected by Taqman PCR after enrichment.
For soil, the $Mobio_{micro}$ method was initially found most efficient. However, this method is based on density separation of bacteria from soil instead of chemical lysis of the soil sample. Due to the experimental setup (addition of *S.* Enteritidis to the substrate prior to DNA extraction), this method would prevent a proper comparison of DNA extraction efficiencies. Therefore, this method was omitted from further experiments.

Some substrates (such as soil S9O and manures M2 and M5) did not allow the detection of *S.* Enteritidis to the same extent as the other substrates tested, irrespective of the DNA extraction method used. It is likely that from these substrates either little DNA was amplified

from the extracted DNA, or the substrates gave a strong inhibition of DNA extraction and amplification. The two manures M2 and M5 were both derived from cattle fed with low digestible grass silage, resulting in manure of high dry-matter. As the feed of cattle has a direct influence on the composition of their manure, the inhibiting components might have originated from the preserved grass. Also, due to the high dry-matter content of these manures (Franz *et al.*, 2005) it is likely that per 100 mg of manure a higher concentration of inhibiting agents is included in the DNA extract, leading to a reduction in extracted DNA yield and/or leading to inhibited amplification. Unfortunately the chemical and / or organic components present in manure M2 and M5, which were responsible for the reduction in DNA extraction efficiency, could not be identified by gas chromatography-mass spectrometry. The inhibiting components should be identified to allow the development of more generic DNA extraction methods for extraction of DNA from complex substrates. Nevertheless, from these data it is evident that in some cases alternative approaches for DNA extraction are required to prevent poor DNA recovery or presence of co-extracted amplification inhibitors. Recognition of the exceptions that lead to insufficient DNA extraction efficiency was previously not possible without extensively studying the efficiency. This major drawback is now countered by application of the GIPC with the substrate prior to DNA extraction.

Despite the clear differences among test kits in extraction efficiency, testing of DNA extraction from substrates inoculated with *S.* Enteritidis suspension does not completely reflect real environmental samples containing naturally present *S. enterica.* In fact, naturally present *S. enterica* might be aggregated on or between substrate particles, which could make the extraction of DNA from all cells present even more complicated. To what extent spatial distribution affects the *S. enterica* DNA extraction efficiency has not been evaluated. Nevertheless, based on the rigorous lysis and homogenisation during the DNA extraction procedures it is expected to have only minor influence on the recovery of *S.* Enteritidis DNA present in the tested substrates.

Direct evaluation of the efficiency of the complete DNA extraction and amplification procedure was enabled by implementation of a general internal procedural control (GIPC). This improvement enabled the identification of false negative results introduced by procedural failures or mistakes. A major advantage of the developed GIPC is the fact that it is absent in natural environments (except in the cnidarian jellyfish *Aequorea victoria*), since its detection is based on the *gfp*-gene of the GIPC. Therefore, independent of the substrate tested, the *gfp*-gene can be detected simultaneously with the target (in our case *S.* Enteritidis) after DNA extraction using Taqman PCR. This is an advantage over other previously published approaches, which use housekeeping genes to control the DNA

extraction and amplification efficiency. The use of such housekeeping genes is not sufficient, since the exact initial amount of control material (DNA) is not known, thus allowing only qualitative validation of a sample tested. Moreover, these controls are only applicable if the corresponding housekeeping genes are indeed present in the environmental sample (plant, animal cells) tested. Finally, the amount of housekeeping genes often exceeds the amount of target DNA, resulting in a competitive PCR amplification strongly affecting the sensitivity, precision and accuracy of the assay. The co-amplification of a different target will in each case affect the sensitivity of the primary target amplification. To reduce any influence of co-amplification the amount of GIPC is limited to a set level to ensure that the target DNA is always present in excess. Herewith the amplification of the target is only influenced at a very minor level (Klerks *et al.*, 2004).

In general, a more accurate quantification of *S. enterica* (or any other pathogen) from soil, manure or compost can be obtained using the GIPC. Doing so, first the presence of inhibitory factors is determined by calculating the mean and S.D. of the GIPC Ct-values. Samples that present a statistically different Ct-value (of GIPC) than the water controls and the negative substrate control samples are not valid for quantification. From all other samples the amount of target CFUs can then be calculated.

Applying this approach, a more accurate quantification of the target initially present in the substrate tested was allowed using a dilution series of *S.* Enteritidis added to soil and manure. However, these data also indicated that the applied molecular approach (DNA extraction followed by Taqman PCR) still brings quantification errors when detecting an organism like *S. enterica* in environmental substrates. Until a (near) perfect quantification of organisms (or a specific DNA target from environmental substrates) is feasible using molecular techniques, the approach described in this paper might provide a more accurate quantification of target organisms / DNA compared to other currently used molecular quantification methods. Finally, the method presented in this paper might be a good addition to the standardised methods for identification and detection of *S. enterica* in environmental substrates, especially since pre-enrichments of samples are currently still obligatory to enable the detection of even 1 CFU of *S. enterica* in 25 g of substrate.

In conclusion, the optimised procedure provides an improved, sensitive and precise method, eliminating false negative diagnosis due to introduction of the general internal procedural control (GIPC). The approach of adding a fixed amount of GIPC prior to DNA extraction provides an efficient and reliable way for evaluating and validating DNA extraction and amplification of each individual sample in one tube, independent of the substrate tested. The

method is applicable for high throughput analysis and routine diagnosis and allows a more accurate quantification of *S. enterica* present in soil, manure or compost.

Acknowledgements

This research was supported by the Horticultural Product Board (Produktschap Tuinbouw), the Netherlands, and the Technology Foundation STW, applied science division of NWO, the technology program of the Ministry of Economic Affairs, and the NWO Russia collaborative grant 047.014.001. The authors would like to thank Dr. H. Aarts from RIKILT, the Netherlands for providing *S.* Enteritidis ATCC 13076 and Dr. R. J.Sayler (Department of Plant Pathology University of Arkansas. Fayetteville, AR 72701 USA) for providing the *E. coli* strain 99507*gfp*. The authors would also like to thank E. Franz, MSc and Dr. A. Termorshuizen for the constructive discussions and their valuable statistical expertise.

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Chapter 4

Physiological and molecular response of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin

Applied and Environmental Microbiology (2007) *Accepted*

Physiological and molecular response of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin

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Summary

This paper describes the physiological and molecular interactions between the human pathogenic *Salmonella enterica* serovar Dublin and the commercially available mini Roman lettuce cv Tamburo. The association of *S.* Dublin with lettuce plants was first determined, indicating the presence of significant populations outside and inside the plants. The latter was evidenced from significant residual concentrations after highly efficient surface disinfection (99.81%) and from fluorescence microscopy of *S.* Dublin in cross-sections of lettuce at the root-shoot transition region. The plant biomass reduced significantly compared to that of non-colonized plants upon colonization with *S.* Dublin. Next to the physiological response, transcriptome analysis by cDNA-AFLP also provided clear differential gene expression profiles between non-colonized and colonized lettuce plants. From this generally and differentially expressed genes were selected and identified by sequence analysis, followed by RT-PCR displaying the specific gene expression profiles in time. Functional grouping of the expressed genes indicated a correlation between colonization of the plants and an increase in expressed pathogenicity-related genes. This study indicates that lettuce plants respond to the presence of *S.* Dublin at a physiological and molecular level as shown by the reduction in growth and the concurrent expression of pathogenicity-related genes. In addition, it was confirmed that *Salmonella* can colonize the interior of lettuce plants, thus potentially impose a human health risk when contaminated lettuce is processed and consumed.

Introduction

In recent years an increase in bacterial foodborne disease outbreaks has been associated with the consumption of uncooked vegetables (i.e. Anonymous, 1999; Fisher, 2004; Hilborn *et al*., 1999; Lyytikainen *et al.*, 2004; Michino *et al.*, 1999). The economic impact of these outbreaks is large, for example each year *Salmonellosis* is responsible for 3.5 million cases in the US and Canada, leading to economic losses up to 3.4 billion $ a year (Todd, 1989). Especially bacterial pathogens like *Salmonella enterica* (Guo *et al.*, 2001), *Escherichia coli* O157:H7 (Solomon *et al.*, 2002), *Bacillus cereus* (Beuchat and Ryu, 1997), *Listeria monocytogenes* (Robertson *et al.*, 2002), *Campylobacter jejuni* (Beuchat and Ryu, 1997), and *Pseudomonas* spp. (Hamilton-Miller and Shah, 2001; Viswanatan and Kaur, 2001) are of major concern due to the environmental occurrence of these bacteria. The presence of human pathogenic bacteria has been described on a wide range of plant hosts (Beuchat and Ryu, 1997; Guo *et al.*, 2001; Guo *et al.*, 2002; Hamilton-Miller and Shah, 2001; Hilborn *et al.*, 1999, Natvig *et al.*, 2002; Robertson *et al.*, 2002; Solomon *et al.*, 2002; Viswanatan and Kaur, 2001; Zenkteler *et al.*, 1997). For greenhouse grown vegetables, these pathogens are suggested to be introduced as a result of bad hygiene during the production or post-harvest processing of the crops (Anonymous, 1999; Beuchat and Ryu, 1997). However, contamination of vegetables may already occur in the field when manure is used for soil fertilization before planting the seedlings (Natvig *et al.*,2002; Viswanatan and Kaur, 2001). Manure is known to harbor high numbers of human pathogenic bacteria like *S. enterica* and *E.coli* O157, which can remain viable for extensive periods of time, even up to one year (Baloda *et al.*, 2001; Kudva *et al.*, 1998; Wang *et al.*, 1996). Even when applying artificially contaminated manure to soil, the number of enteric bacteria was reduced only 1 order of magnitude after a period of three months (Franz *et al.*, 2005). Thus, contamination of plants with human pathogenic bacteria from manure may occur, for example during rainfall or irrigation due to splashing of soil and bacteria onto the plants (Natvig *et al.*, 2002).

Alternatively, plants could be colonized via the roots in manure-amended soil (Solomon *et al.*, 2002; Zenkteler *et al.*, 1997). The colonization of plants via the roots by human pathogenic *E. coli* was observed using a gfp-tagged strain of *E.coli* O157:H7 that colonized the interior of lettuce from soil via the roots up to the leaves (Franz *et al.*, 2007; Solomon *et al.*, 2002). In contrast to this, two other studies found *E.coli* O157:H7 not able to colonize the edible parts of spinach (Hora *et al.*, 2005) or crisphead lettuce (Johannessen *et al.*, 2005), although the bacteria were detected in the rizosphere and on the root surface. With respect to *Salmonella*, gfp-tagged strains colonized the interior of tomato plants when grown

hydroponically (Guo *et al.*, 2001; Guo *et al.*, 2002). Also, an avirulent strain of *S.* Typhimurium colonized carrots and radishes which were grown on a field treated with contaminated manure composts or irrigation water (Islam *et al.*, 2004). Just recently, *S.* Typhimurium LT2 and DT104h were found to endophytically colonize barley sprouts during growth in an axenic system (Kutter *et al.*, 2006). FISH analysis of radial slices indicated the presence of *S.* Typhimurium inside the plant tissue.

However, only very few studies have investigated the physiological effect or molecular interaction between human bacterial pathogens and a plant host, i.e. the model plants *Medicago* and *Arabidopsis*. On *A. thaliana* it was shown that the opportunistic human pathogen *Pseudomonas aeruginosa* PA14 attached to the leaf surface, congregated at the stomata or wounds, and then invaded the leaves and colonized the intercellular spaces (Plotnikova *et al.*, 2000). The bacterium was also able to make circular perforations in mesophyllic cell walls to allow penetration. From this study it was concluded that *Pseudomonas aeruginosa* PA14 is a facultative pathogen of *A. thaliana* that can cause local and systemic infection, eventually leading to plant death. Also mutants of the human pathogenic *Staphylococcus aureus* (Prithiviraj *et al.*, 2005) that contained disrupted genes involved in animal pathogenesis, were attenuated in their ability to infect *A. thaliana*. This suggested that the same regulators that mediate synthesis of virulence factors essential for animal pathogenesis are also required for plant pathogenesis (Prithiviraj *et al.*, 2005). Resistance of *A. thaliana* to *S. aureus* was mediated by a direct effect of salicylic acid on the pathogen affecting attachment on the root surface and reducing pathogen virulence.
Different *Salmonella* serovars were able to endophytically and epiphytically colonize *M. sativa* (Dong *et al.*, 2003). A recent study revealed that colonization of *M. truncatula* by *S.* Typhimurium resulted in the induction of salicylic acid –dependent and –independent plant defenses (Iniguez *et al.*, 2005). Tthe induction of both plant defense pathways was correlated to the bacterial gene expression of TTSS-SPI effector proteins, whereas the presence of flagella only induced the SA-dependent plant defense induced by expression of the PR1-gene. Although these studies point at a specific host-pathogen interaction, until now no research has been described studying the gene-expression of plants during colonization by human pathogenic bacteria, such as *Salmonella*.

The objectives of this study were to investigate the physiological and molecular response of *L. sativa* by *S.* Dublin during plant colonization. Colonization of lettuce plants by *S.* Dublin was studied by comparing the prevalence and the degree of colonization on surface-disinfected and untreated plants grown in nutrient water-agar and in manure-amended soil. Epiphytic and endophytic presence of *S.* Dublin was investigated to provide insight in the

capability of *S.* Dublin to invade plant tissue and to proliferate in or on the plant. To reveal generally and differentially expressed genes upon colonization of lettuce with *S.* Dublin in time, cDNA-AFLP gene-expression profiling was studied. Transcript derived fragments were subjected to sequence analysis and grouped by gene function. Subsequent gene-expression profiling of selected genes was performed using RT-PCR.

Materials and Methods

Plant material and bacterial strains

Seeds of *Lactuca sativa* cultivar *Tamburo* (mini-Roman lettuce) were kindly provided by Mr. Raats (Nickerson-Zwaan BV, The Netherlands). The seeds were surface-sanitized by washing with 1% sodium hypochloride / 0.01% Tween 20, and water (twice), for 1 min each. Subsequently the seeds were air-dried for 1 hour.

A liquid culture of *S. enterica* serovar Dublin grown overnight at 30°C in tryptic soy broth, was kindly provided by Dr. H. Aarts (RIKILT, The Netherlands). The culture was maintained by both plating on selective Hektoen enteric agar (Biotec Laboratories Ltd., UK) and overnight incubation in buffered peptone water (BPW) at 37°C. An *E. coli* JM109 culture (obtained from the collection of Plant Research International BV) was maintained on Luria Broth (LB) plates and in liquid LB medium by overnight incubation at 37°C.

Surface disinfection of lettuce plants colonised with Salmonella *Dublin*

To determine the efficiency of surface disinfection, 35 six weeks-old lettuce plants (grown on soil) were inoculated with 20µl of $2x10^8$ CFU / ml of *S.* Dublin. In total 10 µl of the inoculum was spread across the surface of one leaf and 10 µl was spread across the bottom of another leaf. After 5 min incubation at room temperature the plants were cut at the transition point and from 25 plants the leafy parts were disinfected by rinsing for 10 sec in 70% ethanol and twice in water. Subsequently, each plant (leafy part) was ground in 1 ml of BPW and a dilution series (100x and 1000x diluted) was prepared from the suspension. Each dilution was plated (40µl) on Hektoen enteric agar, in duplicate, and incubated overnight at 37°C prior to colony counting. The means and standard errors of the number of *Salmonella* CFUs recovered were calculated, and the surface disinfection efficiency was determined by the ratio between the mean [number of CFUs recovered from surface-disinfected plants] and the mean [number of CFUs recovered from non-disinfected plants].

Association of Salmonella *Dublin with lettuce grown in manure-amended soil*

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 mg / kg and 22400 mg / kg, 11% moisture and had a pH of 7.14. The manure contained 28.7% acid detergent fibre, 40.3% neutral detergent fibre, a total dissolved organic N and C of 740 mg / kg and 8167 mg / kg, 220 mg / kg ammonium, 8.14 mg / 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 1g of each substrate using real-time PCR analysis (Klerks *et al.*,2004).

Manure was inoculated with 10^8 CFU of *S.* Dublin / g wet weight and mixed thoroughly before addition to soil at a weight ratio of 1:10. The final number of *S*. Dublin CFU was10^7 / g fresh mixture. In total 74 pots of 50 ml with 50 g of *S.* Dublin contaminated soil / manure mixture were prepared. The negative control pots (74 in total) contained non-*S*. Dublin-inoculated manure / soil mixture. One lettuce seed was added to each pot (148 in total) and allowed to germinate in a greenhouse at 18°C and 80% humidity. After 6 weeks, each plant was harvested by cutting the plant at the stem just above the soil. The plants were each weighed and thoroughly washed in 30 ml of sterile water prior to analysis. Next, for both treatments all plants were randomly divided in two sets of 38 plants. Each plant of the first set of plants was ground in 1 ml of BPW. From the second set of plants each plant was surface disinfected as previously described, followed by grinding in 1 ml of BPW. Each suspension of ground plant material was plated (40µl) on Hektoen enteric agar, in duplicate. In addition, the wash fraction was centrifuged and the pellet resuspended in 100µl of BPW prior to plating on Hektoen enteric agar, in duplicate (40 µl / plate). After overnight incubation at 37°C the total number of *Salmonella* CFUs was counted for each plate. To determine a significant difference in plant weight between both treatments, a paired t-test was performed for all tested plants per treatment.

Association of Salmonella *Dublin with lettuce grown on Hoagland's agar*

Sterilized lettuce seeds (120) were allowed to germinate for 3 weeks on 0.5% Hoaglands agar (pH 6.8) in closable growing units (10 x 15 x 8 cm) placed in a growth chamber at 20°C with 12 hrs light / dark intervals. To assess the colonization of lettuce by *S.* Dublin over time, the 120 three weeks-old lettuce plants were inoculated at the root site with 10µl of 10^7 CFU / ml of *S.* Dublin, without wounding the roots. Every two days for a period of twenty days, the shoots of 12 lettuce plants were cut off just above the agar and weighed. To determine the

prevalence, degree of colonization, and localization of *S.* Dublin (endophytically or epiphytically) associated with lettuce plants, the harvested leafy parts of six plants were not surface disinfected whereas leafy parts of the other six plants were surface disinfected as described earlier. The leafy parts were ground in 0.5 ml of BPW. A dilution series (non-diluted, 10x or 100x diluted) was prepared from each leaf suspension and 40µl of each dilution was plated onto Hektoen enteric agar, in duplicate. With both treatments (with and without surface disinfection) the prevalence and the degree of colonization was determined by calculating the mean *S.* Dublin CFU diminished by the error of surface disinfection efficiency.

Lettuce response to bacterial colonization

The response of lettuce plants to colonization by *S*. Dublin was compared to that by *E. coli* JM109. Water-inoculated plants were used as controls. Thirty seeds were sprouted on sterile Hoagland agar in separate tubes in a closable growing unit (50 x 30 x 25 cm) for 3 weeks in a growth chamber at 20°C and 80% humidity. Next, 10 seedlings were carefully inoculated at the roots with 10µl of 10^7 CFU / ml of *S.* Dublin, 10µl of 10^7 CFU / ml of *E. coli* JM109, or inoculated with water. After 5 weeks, each surviving plant per treatment was cut at the transition point between stem and roots and weighed. To determine significant differences between the treatments with respect to plant death, non-parametric analysis (Kruskal-Wallis test with asymptotic significance) were performed based on the number of surviving plants and the weight of these plants.

Preparation of plant tissue cross-sections

From a different set of lettuce plants that were inoculated in a similar manner as described above, 3 weeks after inoculation with *S.* Dublin the shoot-root transition region was cut for microscopic analysis before cross-sectioning (in total 1 cm of transition region was obtained for each plant). First, the cut transition regions were incubated overnight in fixative (ethanol 96% : acetic acid (3:1 v/v)). After fixation the tissues were transferred to a graded series of sucrose solutions with increasing concentrations of 5, 10, 20, 30, 40 and 50% (w/v) in PBS (phosphate buffered saline, pH 7.4). Samples were kept in each concentration for 30 min. Next, the samples were embedded in Tissue-Tek O.C.T compound (Miles, Elkhart, In) while ensuring a vertical position of the samples. Cross sections of 20 µm and 200µm thickness were cut from each sample using a cryostat (Microm, HM 500 O, Microm Laborgeräte GmbH, Walldorf, Germany) at -30°C. The tissue sections were transferred to poly-l-lysine (Sigma Chemicla Co, St. Louis, MO, p-1524; 0.1% in milliQ (w/v)) coated slides and dried at 60°C for 15 min and stored at -20°C for further use. To label the potentially present *S.* Dublin in each cross section, the slides were first incubated at 70°C for 15 min, washed once with

milliQ (without shaking) at RT, dried at 70°C, washed twice with milliQ at RT, dried at 70°C, washed with PBST (PBS including 0.1% Tween) for 2 min at RT, washed with PBS with 2% bovine serum albumin (BSA) for 10 min and washed twice with PBST at RT. Next, the cross sections were incubated in the dark for 60 min at RT with 200 µl of label-mix, which consisted of PBS pH7.4, 10 µg of Fluorescein (FITC)-labeled polyclonal antibody to *Salmonella* common structural antigens (KPL Europe, Guildford UK) and 10µl of FA Rhodamine counterstain (Difco Laboratories, Detroit, USA). After staining, the cross sections were washed three times with PBS, pH 7.4, before 100µl of mounting solution (Vectashield mounting solution for fluorescence; Vector Laboratories, Inc., Burlingame, CA) was added and slides were covered and sealed. Each slide with cross-sections was analyzed using a fluorescence microscope including CCD-camera.

Messenger RNA and DNA preparation for gene-expression analysis

For gene-expression analysis, 60 seeds were germinated in Hoagland's agar, of which after 3 weeks growth 30 plants were inoculated close to the roots with 10 µl of water and 30 plants were inoculated with 10 µl of 10^7 CFU/ml *S.* Dublin. Every two or three days for a 3-week period (10 time points, including t=0) three water-inoculated plants and three S. Dublin-inoculated plants were harvested by cutting the plants just above the agar surface. At each time point the leafy parts of each treatment were pooled prior to weighing and subsequently ground in liquid nitrogen and stored at -80°C. Total RNA was extracted from the ground samples using Qiagen Plant RNeasy kit (Westburg, Germany) according to the supplier's protocol (including DNase treatment). The total RNA eluates were aliquoted in several portions before storing at -80°C. Plant mRNA was purified from 45µl of each total RNA sample using the Oligotex mRNA purification kit (Invitrogen). For DNA extraction from ground plant material, the Qiagen plant DNeasy kit (Westburg) was applied as described by the supplier's protocol. The purified DNA was dehydrated / dried using a speed vacuum concentrator, re-suspended in 50µl of milliQ water and stored at -20°C until further use.

cDNA-AFLP differential gene-expression analysis of S*. Dublin colonized lettuce*

Basic principles of *c*DNA-AFLP were followed as described by Bachem *et al.* (1996; 1998). Each first strand cDNA synthesis reaction was prepared by incubating 10µl of purified mRNA with 2µl of polydT primer (5µM) for 10 min at 70°C, followed by incubation on ice. Next, 4µl of first strand buffer (Invitrogen), 2µl 0.1M DTT, 1µl of 10mM of each dNTP was added to each RNA sample and shortly incubated at 37°C. First strand synthesis was started by adding 1µl of Superscript II RT RNase H^- to each reaction. The sample was incubated for 1 hr at 37°C before ending the synthesis by incubation on ice.

Second strand synthesis was performed for each first strand reaction in a total volume of

150µl containing (Invitrogen), 500nmol of each dNTP, 10 units of *E. coli* DNA ligase, 40 units of *E. coli* DNA polymerase I and 2 units of *E. coli* RNase H, followed by incubation of 2 hrs at 16°C. Then, 2µl of T4 DNA polymerase was added followed by a short incubation of 5 min at 16°C. The final ds cDNA was purified by phenol:chlorophorm:iso-alyl alcohol (1:1:24) and NaAc precipitation. The precipitated DNA was resuspended in 25µl of water.
To perform restriction digestion of the prepared cDNA, 10µl of cDNA sample was added to 40 µl of restriction mix, containing a final concentration of 1xRL-buffer with 10 units of *Mse*I and 50 units of *Eco*RI. The mix was incubated overnight at 4°C.
Subsequently, 20µl of cut cDNA sample was added to 30µl of ligation mix, with a final concentration of 5pmol of *Eco*RI-adapter, 50pmol of *Mse*I-adapter, 1xT4 DNA ligase buffer (Invitrogen) and 5 units of T4 DNA ligase. Ligation was performed by incubating the ligation sample for 90 min at 37°C. The ligated cDNA samples were stored at -20°C upon further use.

Pre-amplification was performed using primers directed to the adapters that were ligated to the cDNA (zero-reaction). Each PCR reaction of 25µl consisted of PCR buffer (10 mM Tris/HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 150nmol of each dNTP, 63pmol of each primer EcoRI00 (5' GACTGCGTACCAATTC 3') and primer Mse00 (5' GATGAGTCCTGAGTAA 3'), 1 unit of Taq polymerase (Gibco BRL) and 5µl of ligated cDNA sample. The reaction mix was incubated for 2 min at 94°C, followed by 35 cycles of respectively 30 sec at 94°C, 30 sec at 56°C and 90 sec at 72°C.

Selective PCR was performed as described above, using the primers 33P-kinated EcoRI19 (3' +2 overhang; GA) and Mse11 to Mse26 (+2 nucleotides 3' overhang, each possible combination) with 5 µl of a 50 times dilution of pre-amplification product in a total volume of 20µl. Primer EcoRI19 was kinated to gamma-33P-ATP using polynucleotide kinase prior to PCR. The PCR profiles were as follows: 1 cycle of 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, followed by 12 cycles of 94°C for 30 sec, 65°C [-0.7°C/cycle] for 30 sec, 72°C for 1 min, and 23 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min. PCR products were size fractionated on a 5% poly-acrylamide gel and run for 1.5 hrs at 80W.

Isolation of differentially expressed gene fragments and confirmation by RT-PCR

AFLP gels were vacuum blotted and dried on Whatmann 3MM paper for 1 hr and subsequently exposed to X-ray films for 2 weeks. After film development bands of interest were selected and cut from the blotted gel on Whattman paper. The small paper cuttings were stored in a microtiterplate with 100µl of RNase free water and heated for 5 min at 95°C

to elute the DNA from the paper. Next, 5 µl of eluted sample was amplified again by PCR (according to the zero-reaction protocol). Samples were analyzed on gel and sequenced using primer E00. Each transcript-derived fragment (TDF) sequence was compared against all sequences in the non-redundant database using the tBlastX program with the EMBL Database, and TIGR EST library of *L.sativa* and *A. thaliana*.

Table 1. *Primer sequences for RT-PCR of plant genes*

primer	Sequence 5' – 3'	Gene homology	Acc. number[a]	p
FPPR1	GGTACACGGCTTATGGTCAAACAG	Pathogenicity-related protein 1	BQ846446	0
RPPR1	TCCATAAGCCACCAAATCAGCATC			
FPPR4	GATCTCTTAGCCACAAACCCAACC	Pathogenicity-related protein 4	BQ874271	0
RPPR4	AACCGGACCCGCTGACCTATCT			
FPPR5	TGCCTCGGAGATTAGTGGGGATAG	Pathogenicity-related protein 5	BQ869968	0
RPPR5	CGCCGTCAATACCGCTTTTACA			
FPDAD1	GACGGCGACGACGAAAGATGAT	Defender against apoptotic death protein 1	BQ987261	0
RPDAD1	GCGGTGAAGACGGCGAACA			
FPTHRE	GCTATAGGCCTTGCTGCTGTTCTC	Probable threonine ammonia-lyase	BQ870155 / TC12830	$8.5e^{-14}$
RPTHRE	GGTTTCATGGGCCTCCTTATTT			
FPEXP	GGAATCACATCCCTTGCTGACAGA	Beta-expansin 1 precursor (At-EXPB1) (Ath-ExpBeta-1.5)	BQ867493	$6.6e^{-18}$
RPEXP	TAACCGCGGCGTACTGAACATC			
FPOXY	ACAGCTCCACCCGTTTGACACC	Oxygen-evolving enhancer protein 3-2 chloroplast precursor (OEE3)	BQ995404 / TC12174	$6.6e^{-36}$
RPOXY	TCTTTCGCCGATTCCTTTACACG			
FPREC	GCAAGGACCAGTAGGCGAGGTGTAC	Receptor protein kinase-like protein	BQ867195	$1.8e^{-09}$
RPREC	ACAACCCCAAAAGAATAAACATC			
FPNAM	TCAAGTCCCGGAAGTAAAAGAG	NAM-like protein	BQ864249 / TC12743	$1.9e^{-05}$
RPNAM	ACCTGATGATGGATAAGAAATAGC			
FPPAT	TCCGACGTCAAAAAGAAGATAAC	Pathogenesis-related protein 1 precursor (PR-1)	BQ846446	0.95
RPPAT	CTTACACACACATATTCATTCA			
FPPYR	TCGAAGGCTCCGGTGATAAAAT	pyruvate dehydrogenase kinase	BQ870017 / TC11537	$6.4e^{-29}$
RPPYR	NTGAGAAAGGGTTGCGTGTTG			
FPPHO	GCCCCTAAAACCCCTCCTCT	phospholipid hydroperoxide glutathione peroxidase	TC9259	$1.4e^{-05}$
RPPHO	AACCCCTCCTTCTAGCGATTCA			
FPBHL	CCGAACGGAAAAGAAGAGACAAG	putative bHLH transcription factor bHLH016	TC9488	$1.6e^{-07}$
RPBHL	GTGGACCACAGGTTTGATTTTGC			
FPSEC6	TGATAAAGTCCAGCCTCCAAAAT	SEC6	TC14586	0.86
RPSEC6	GCAAGATCATAGCATCTCAAGTTGT			
FPSEC1	AATGGTTGAATCCGCGTTGAGAG	SEC1-family transport protein SLY1	TC9658	$1.0e^{-12}$
RPSEC1	TTAGGCAGGAGCAGAAGCAGAAGG			

[a] *Accession numbers of genes in TIGR gene indices, that is linked to the non-redundant EMBL database*

[b] *The smallest sum probability (p-value) according to EMBL genbank*

To allow gene confirmation and expression profiles by RT-PCR, primer sets were designed based on alignments of TDFs with the most probable sequence hits from the EMBL database and TIGR-EST database. Each primer set was designed such that one primer was located inside both sequences of the alignment, and one primer was located outside the TDF, but inside the sequence obtained from the TIGR-EST database. Next to that, other primer sets were designed based on specific lettuce genes related to plant-pathogen interaction, namely pathogenicity-related gene1 (PR1; Coquoz *et al.*, 1995), gene 4 (PR4; Ruperti *et al.*, 2002) and gene 5 (PR5; Uknes *et al.*, 1992) and defender against apoptotic death (DAD-1; Nakashima *et al.*, 1993). All primer sequences and corresponding genes are displayed in Table 1.).

In each case the first strand cDNA synthesis was performed using the reverse primer and MuLV-reverse transcriptase. Each 30µl target-specific PCR-reaction consisted of PCR buffer (10 mM Tris/HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 100nmol of each dNTP, 167pmol of each target-specific primer, 1.25 units of Taq polymerase (Gibco BRL) and 0.25 µl of cDNA sample. The reaction mix was incubated for 2 min at 94°C, followed by 35 cycles of respectively 15 sec at 94°C, 30 sec at 58°C and 1 min at 72°C and finished at 10°C for 10 min. All amplification products were analyzed by electrophoresis using a 1% pronarose gel containing 0.5 µg / ml of EtBr. The intensity of the resulting bands was normalised for each gene-specific primer set using the most intensive band as 100% expression level to allow proper comparisons of the time series of both non-inoculated and inoculated samples.

Results

Plant surface disinfection efficiency

To investigate the presence of *S.* Dublin inside plant tissues, the Salmonellae on the plant surface must be removed very efficiently without killing the bacteria inside the plant. To this extent the efficiency of ethanol 70% was evaluated for surface disinfection of the leafy parts of plants that were inoculated directly on the leaves with *S.* Dublin. On average 5.6 (±1.0) $x10^3$ *S.* Dublin CFUs were recovered after disinfection and 2.9 (±0.1) $x10^6$ CFUs were obtained when no disinfection was applied to the *S.* Dublin-inoculated leaves. From these results the surface disinfection efficiency was determined as 99.81% (± 0.26%).

Colonization of lettuce grown in manure-amended soil by Salmonella *Dublin*

Since Salmonellae are frequently isolated from bovine manure, it was hypothesized that lettuce plants grown on manure-amended soil can be colonized by these Salmonellae. To investigate the prevalence and degree of infection of lettuce plants with *S.* Dublin, lettuce seeds were applied to soil that was amended with non-inoculated manure or *S.* Dublin inoculated manure. From the seeds applied on soil amended with non-inoculated manure, 61 out of 74 seeds germinated. In soil amended with *S*. Dublin inoculum 56 out of 74 lettuce seeds germinated. This difference was not significant (Chisquare=1.02). The mean weight of the leafy parts of the plants grown for six weeks on *S.* Dublin inoculated manure / soil mixture was 0.52 g ± 0.17 g and the mean weight of the leafy parts of the plants grown for six weeks on non-inoculated manure / soil mixture was 0.57 g ± 0.15 g. No significant difference was observed between both treatments using analysis of variance (p=0.153).

The prevalence of *S*. Dublin found in association with the leafy parts of lettuce plants was 27% (15 out of 56 plants). The wash fraction of 15 sampled plants also contained *S*. Dublin, indicating that 27% of the plants were in each case colonized above soil with loosely attached *S*. Dublin. Moreover, three surface disinfected plants were positive for *S.* Dublin (5%), suggesting the presence of *S*. Dublin inside the plant tissue. From these internally colonized plants in two cases also the wash fraction was positive for *S*. Dublin, which indicated the presence of *S.* Dublin also on the plant surface. The number of *S*. Dublin CFU recovered from the surface disinfected plants ranged from 75 CFU to 1275 CFU per plant. In addition, one non-disinfected plant was also positive for *S.* Dublin, indicating the presence of internal and / or external colonization of the plant by *S*. Dublin. These results suggest that lettuce plants can be colonized by Salmonellae, in our case *S.* Dublin, when grown on soil amended with contaminated manure.

Colonization of lettuce grown in Hoagland's agar by Salmonella *Dublin*

To study the colonization of lettuce by *S.* Dublin in time, lettuce seeds were germinated on Hoagland's agar and after three weeks the plants were carefully inoculated at the roots with *S.* Dublin. The number of plant-associated *S*. Dublin CFU varied greatly over time among colonized plants, which led to a high S.E.. Therefore no correlation between number of *S.* Dublin CFU and time post-inoculation was obtained. (Table 2). Yet, a large difference in total number of *S.* Dublin CFU was found between the disinfected (mean of 3808 ± 1643 CFUs per plant) and non-disinfected plants (mean of 49582 ± 30012 CFUs per plant) (Table 2). Taking into account the surface disinfection efficiency (99.81%), in this experiment a maximum of 94 CFUs (0.19% of 49582 CFUs) was considered false positive among the disinfected plants. This is appr. 40-fold lower than the average number of CFUs found inside

the disinfected plants.

Based on prevalence of *S.* Dublin in association with lettuce plants in the time series tested, all but 3 non-disinfected seedlings were positive (Table 2). In total 17 out of 54 disinfected plants were below the threshold level of 94 CFUs, which indicated that 31% of all 54 disinfected plants was false positive. Still, at least 43% of the 54 disinfected plants were confirmed positive for *S.* Dublin, taking into account the false positive threshold of 94 CFUs.

From these results *S.* Dublin appeared more present at the outer surface compared to the inside of the plants (mean ratio of 13:1). The degree of colonization in time with *S.* Dublin inside the lettuce seedlings ranged from 100 CFU up to $4.4x10^4$ CFU (potential false positives excluded), whereas the degree of colonization both inside and outside the plants ranged from 2 CFU up to $1.1x10^6$ CFU per plant.

Table 2. *Colonization of lettuce by* Salmonella *Dublin over time*

	disinfected[a]			**non-disinfected**[a]		
Sampling day	prevalence[b]	CFU / plant[c]		prevalence	CFU / plant	
		Mean	SE[d]		Mean	SE
0	0/6	0	0	0/6	0	0
2	2/6	297	43	6/6	14803	2641
4	2/6	2094	455	5/6	7756	2851
6	2/6	2413	324	5/6	18139	2919
8	2/6	1678	318	4/6	2377	606
10	2/6	5284	1200	6/6	10624	1895
12	5/6	10403	3118	6/6	6035	979
14	1/6	1463	0	6/6	13843	2347
16	2/6	1138	243	6/6	34070	7305
18	5/6	1661	363	6/6	317531	68920
total	43%	3808	1643	93%	49582	30012

[a] *Surface disinfected and non-disinfected lettuce plants tested for presence of* Salmonella *sp. in time*

[b] *The number of plants positive for* Salmonella *Dublin / total number of plants tested, in time*

[c] *The mean number of* Salmonella *CFU per plant that were in association with the surface disinfected and non-disinfected lettuce plants, in time.*

[d] *The standard error of the mean number of* Salmonella *Dublin CFU found on the plants per timepoint.*

Localization of S. *Dublin in association with lettuce plants*

To evaluate if *S.* Dublin was able to colonize the plant up to the leaves, different plant parts were tested by grinding the tissues and subsequent plating on selective Hektoen agar. From this experiment *S*. Dublin was found associated with lettuce plants, mainly from the root-stem transition point up to the leaves, but not in the leaves (data not shown).

Then, to determine the possible point of entrance of *S.* Dublin in lettuce plants, cross sections of colonized plants were analyzed by fluorescence microscopy (Figure 1). Analyses of the cross-sections revealed strong growth of *S.* Dublin on the root surface (1A and B) and near emerging lateral roots (1C). Moreover, internalization was observed via the intercellular spaces between epidermal cells (1D). *S.* Dublin bacteria were found at the cortex within the parenchyma tissue (1E), either still attached to epidermal cells or spreading further through the parenchyma tissue. In few cases *S.* Dublin was also found attached to endodermal cells, inside the pericycle (1E) or even inside the vascular system (1F), which suggests the bacterium might be able to pass the endodermis and potentially spread upwards via the vascular system.

Symptom development and biomass of lettuce grown in Hoagland's agar

Lettuce plants responded to the presence of *S.* Dublin in and on the plant tissue by showing reduced root formation and stunted growth. With respect to biomass, up to 10 days post inoculation (dpi) no clear differences in plant growth were observed between non-inoculated and inoculated plants (Figure 2). From 12dpi onwards, growth of lettuce plants inoculated at the roots with *S*. Dublin was significantly reduced compared to the non-inoculated lettuce plants. Fitting the data sets using logistic regression, separate curves for the healthy plants (R^2-value of 0.98) and the inoculated plants (R^2-value of 0.97) were obtained (Figure 2).

Linear regression of all data (R^2-value of 0.80) resulted in a worse fit than separate data sets (healthy: R^2-value of 0.95, inoculated: R^2-value of 0.94). A difference in slope (factor 2) of the linear regression curves was obtained which suggests a significantly stronger growth of healthy plants compared to inoculated plants when grown in Hoagland's agar. To determine if the stunted growth was specifically related to the colonization by *S.* Dublin, morphological changes due to the presence of *S.* Dublin were compared to those in the presence of *E. coli* JM109 and to healthy, water-inoculated plants. The non-pathogenic *E. coli* was used to ensure that the symptoms observed were specific for the presence of *S.* Dublin and not due to inoculation side effects or presence of bacteria in general.

From sixteen days post-inoculation onwards, yellow spots appeared on the leaves of 8 *S*. Dublin-inoculated lettuce plants. The root basis of these plants became narrowed just below the transition point from root to stem. At 19 dpi, the leaves were strongly yellowed, while two

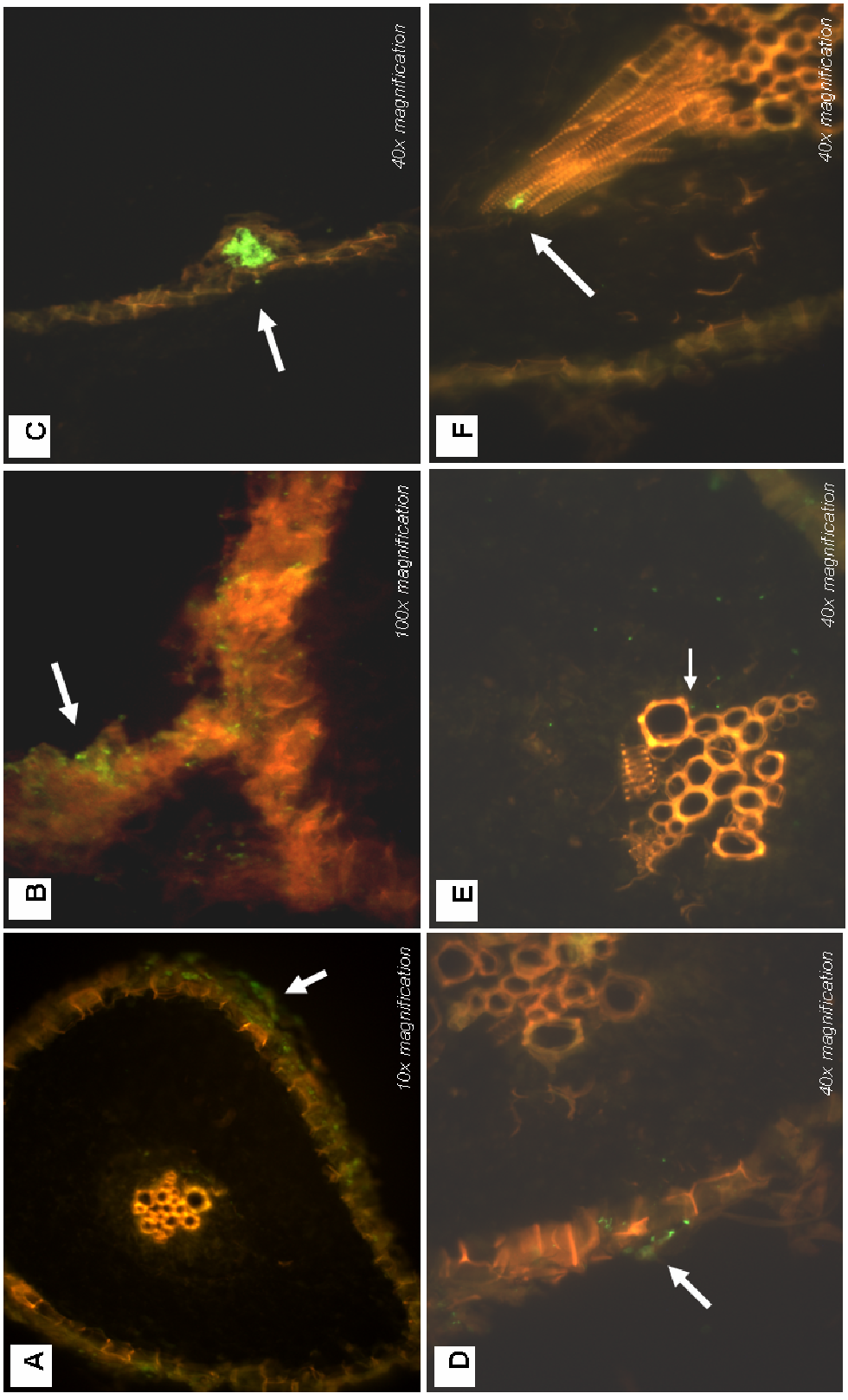

Figure 1. *Fluorescence microscopy of cross sections of the root-shoot transition region of lettuce plants colonized with* Salmonella *Dublin. The S. Dublin was visualized using a FITC-labeled antibody directed to* Salmonella enterica *in the cross sections, indicated by arrows. The bacteria were clearly detected on the root surface (A and B) and at emerging lateral roots (C). Internalization was observed via the intercellular spaces between epidermal cells (D). Endophytically present S. Dublin was observed in the parenchyma tissue (1E), attached to the endodermal cells and inside the pericycle, and inside the vascular system (1F).*

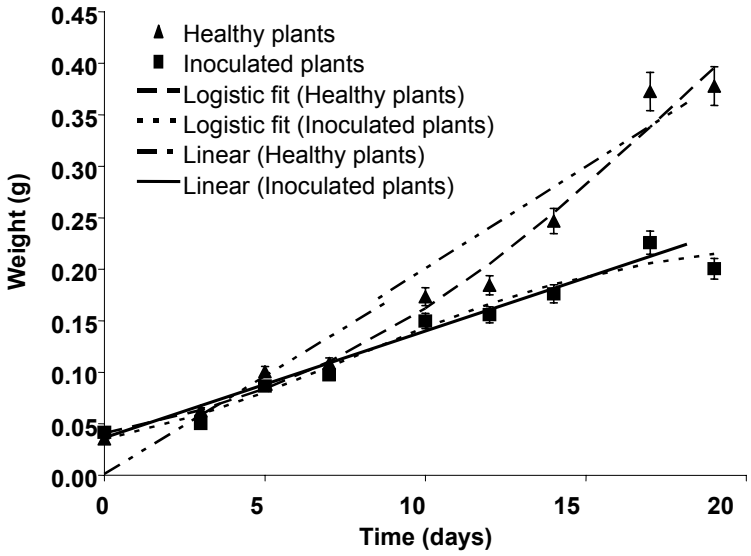

Figure 2. *Logistic and linear regression of plant weight of non-inoculated plants and plants inoculated at the roots with* Salmonella *serovar Dublin versus time of sampling.*

plants remained healthy. However, with *E. coli* JM109 only four plants showed very slight yellowing and stunting at 20 dpi, while six plants remained healthy. During the experiment all ten water-inoculated plants remained healthy. No bacterial growth was detected in Hoagland's agar.

Non-parametric analysis (Kruskall-Wallis test) of the total number of remaining healthy lettuce plants revealed a significant difference between all three treatments (p=0.008). Comparing two treatments for the number of healthy plants, a significant difference (p=0.002) was observed between water-inoculated plants and *S.* Dublin inoculated plants. Water-inoculated plants compared with *E.coli* JM109 inoculated plants showed no significant difference (p= 0.057). This indicated that the observed morphological changes of the lettuce plants were induced by the presence of *S*. Dublin, and not due to the presence of a bacterium in general (in our case *E. coli*) or depletion of nutrients (healthy controls).
As an additional control, the different treatments were also compared based on the biomass of the healthy, i.e. surviving plants, indicating no significant difference (p=0.141) between all plants of the three treatments. This suggested the remaining healthy plants of the three treatments (two *S.* Dublin-treated plants, six *E. coli*-treated plants, ten water-treated plants) were not colonized or influenced by the bacteria added close to the roots.

Identification of lettuce genes differentially expressed due to Salmonella *colonization*

From the physiological response of lettuce plants to *S.* Dublin colonization and the presence of *S.* Dublin in and outside lettuce plant tissues, it was suggested that the plant also responded at a molecular level. To determine the molecular response of lettuce during colonization by *S.* Dublin in time, gene expression analysis was performed using cDNA-AFLP. The transcript-derived fragments (TDFs) displayed from cDNA-AFLP were obtained from 16 primer sets (EcoRI-T and Mse-NN) that were tested. On average for each primer set 100 to150 bands were observed, resulting in approx. 2000 fragments that were analyzed. Although the majority of bands revealed no differential expression profiles between non-inoculated and Salmonella-inoculated plants, also discriminative bands were found. In total 170 bands were selected from cDNA-AFLP, of which 90 bands showed differential expression profiles between both treatments (non-inoculated and inoculated), in time. The selected 170 bands were sequenced of which 68 consisted of more than one sequence, thus leaving 102 sequences valid for further analysis. The 102 sequences were blasted using tblastx against EST databases from *L. sativa* (22.185 EST) and *A. thaliana* (62.010 EST) (TIGR-gene indices). On the basis of sequence homology, these 102 TDFs were grouped in 12 categories of putative function followed by classification according to Mahalingam *et al* (2003) (Table 3).

Comparing differentially expressed TDFs versus generally expressed TDFs, an increase was observed for the categories related to plant disease / defense, transport, signal transduction and hypothetical proteins (table 3). A reduction in gene expression was observed for plant metabolism, and genes with unknown functions. Specific gene-expression due to the presence of *S.* Dublin was confirmed by evaluation of PCR primer sets directed to DNA sequences coding for proteins known to be related to plant stress (Table 1). The expression of the plant stress-related genes DAD1-gene (Nakashima *et al.*, 1993), PR1-gene (Coquoz et al., 1995), PR4-gene (Ruperti et *al.*, 2002) and PR5-gene (Uknes *et al.*, 1992) was induced by the presence of *S.* Dublin at 2dpi (Table 4). This suggested that the plant defense mechanism was activated by the presence of *S*. Dublin in association with the plant.

Next, 11 tentative differentially expressed genes obtained from cDNA-AFLP were analyzed with RT-PCR, of which five genes (Probable threonine ammonia-lyase, Receptor protein kinase-like protein, Beta-expansin 1 precursor, Pyruvate dehydrogenase kinase, and Phospholipid hydroperoxide glutathione peroxidase) eventually appeared to be equally expressed in time with both treatments or did not reveal a significant difference (not shown), and were therefore excluded from further analyses. From the other six genes, namely *NO*

APICAL MERISTEM (NAM)-like protein –gene (Duval *et al.*, 2002), Oxygen-evolving enhancer protein 3-2 chloroplast precursor (OEE3)-gene (Sugihara *et al.*, 2000), pathogenicity-related protein 1 (Coquoz *et al.*, 1995), Secretion 1-family transport protein gene (Aronov and Gerst, 2004), Secretion 6 transport protein gene (Yeaman *et al.*, 2004) and bHLH016 transcription factor protein gene (Heim *et al.*, 2003), differential gene expression patterns were observed between non-inoculated plants and *S.* Dublin-inoculated plants (Table 4). The pathogenicity-related protein1-gene was based on the PR1-gene sequence of *A. thaliana* and gave the same results (as expected) as obtained with the primer set PR1. The NAM-like protein -gene, related to resistance and cell death, revealed an

Table 3. *Contribution of functional groups to general and differential gene-expression*

General[a]			**Differential**[b]			**Difference in expression**[c]
Functional groups	number of genes[d]	%[e]	Functional groups	number of genes	%	
Cell growth/division[f]	1	2%	Cell growth/division	0	0%	=
Cell structure	1	2%	Cell structure	0	0%	=
Disease/defense	6	11%	Disease/defense	9	21%	+
Energy	9	16%	Energy	8	19%	=
Transport	7	13%	Transport	7	16%	+
Metabolism	12	21%	Metabolism	3	7%	-
Protein degradation/storage	1	2%	Protein degradation/storage	1	2%	=
Protein synthesis	1	2%	Protein synthesis	1	2%	=
Signal transduction	3	5%	Signal transduction	5	12%	+
Transcription	1	2%	Transcription	0	0%	=
Hypothetical protein	3	5%	Hypothetical protein	6	14%	+
Unknown	10	18%	Unknown	3	7%	-
Total:	56		Total:	43		

[a] *Generally expressed genes separated by function*

[b] *Differentially expressed genes separated by function*

[c] *Displays a decrease (-), increase (+) or equal (=) number of genes being expressed for each functional group*[f] *when comparing the percentage of generally and differentially expressed genes per functional group.*

[d] *Total number of genes per functional group found after EMBL and TIGR database searching, and* [e] *the percent contribution to the transcriptome*

Table 4. *Gene expression profiles of transcript derived genes and pathogenicity related genes.*

Gene[a]	Treatment[b]	Normalized gene expression in time (dpi)[c]									
		15 min	2	4	6	8	10	12	14	16	18
Defender against cell death-protein-gene	-	0.9[d]	0.8	0.7	0.6	0.6	0.7	0.3	0.4	0.7	0.8
	+	0.8	1.0	1.0	0.8	1.0	0.6	0.6	0.5	0.4	0.2
Pathogenicity related protein 1-gene	-	0.3	0.3	0.4	0.4	0.6	0.7	0.5	0.2	0.3	0.4
	+	0.8	1.0	1.0	1.0	0.6	0.2	0.8	0.4	0.0	0.2
Pathogenicity related protein 4-gene	-	0.2	0.3	0.5	0.5	0.3	0.3	0.8	0.3	0.5	0.8
	+	0.3	0.7	0.3	0.7	1.0	0.7	0.7	0.5	0.3	0.3
Pathogenicity related protein 5-gene	-	0.0	0.3	0.6	0.5	0.3	0.3	0.8	0.3	0.4	0.8
	+	0.6	1.0	0.4	0.6	0.8	0.4	0.6	0.4	0.2	0.4
No apical meristem-like protein-gene	-	1.0	1.0	1.0	0.8	1.0	1.0	0.4	1.0	1.0	1.0
	+	0.3	1.0	0.7	0.3	0.7	1.0	0.3	0.0	0.0	0.0
Oxygen evolving enhancer protein-gene	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.0
	+	1.0	0.7	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sec1 transport protein-gene	-	0.2	1.0	0.8	0.8	1.0	0.6	0.6	0.6	1.0	1.0
	+	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sec6 transport protein-gene	-	0.8	0.8	1.0	0.8	0.8	0.8	0.6	1.0	1.0	1.0
	+	1.0	1.0	1.0	1.0	0.3	0.3	0.5	0.3	0.0	0.0
bHLH16 transcription factor protein-gene	-	0.4	0.4	0.6	0.6	0.6	0.8	0.8	1.0	1.0	1.0
	+	1.0	1.0	0.9	0.9	0.7	0.4	0.4	0.1	0.0	0.0

Gene-expression in time measured by Reverse Transcriptase-PCR for various genes with Salmonella *Dublin-inoculated plants (+) and water-inoculated plants (-). The level of expression was normalized for each gene ranging from high (white) to no expression (black).* [a]*Genes analyzed with for level of gene-expression, in time*
[b]*Treatment of plants that were analyzed in time. Plants were inoculated at the roots with* S. *Dublin (+) or with water (-).* [c]*Sampling times of lettuce plants for gene-expression analysis.* [d]*Normalized level of gene-expression obtained after Reverse Transcriptase-PCR and gel analysis. Normalization was performed for each gene separately based on band intensity on gel.*

increase in expression only at 2 dpi followed by a reduction in expression compared to the NAM-gene expression of non-inoculated plants. The expression of the OEE3-gene (energy function) was consistently high until 6 dpi, but then dropped dramatically in time to undetectable levels. The sec1 and sec 6 transport protein genes are both involved in intracellular mRNA transport and cell proliferation, which each showed a consistent expression in time with the untreated plants. But with the *S.* Dublin inoculated plants, the

gene expression of both genes was reduced to undetectable levels after 4 dpi and 10 dpi, respectively. In addition to this, the expression of the bHLH016 transcription factor protein gene suggested a high level of expression during the early stages of colonization, but slowly reduced in intensity over time. For non-inoculated plants, the expression of this gene was moderate at first, increasing in intensity over time.

Discussion

This study investigated the physiological and molecular response of *L. sativa* cultivar Tamburo to *S*. Dublin. Lettuce plants were colonized both endophytically and epiphytically when lettuce seeds were germinated on *S.* Dublin-inoculated manure-amended soil (prevalence of 27%). Lettuce grown under sterile conditions was even more susceptible to colonization by *S.* Dublin via the roots (prevalence of 43%) than when grown on soil. With both approaches *S*. Dublin was mainly present on the plant surface but also endophytically, at a ratio of 13:1.

These results rely on the surface disinfection efficiency obtained from artificially inoculated leaves. This does not fully reflect a completely realistic situation. Naturally occurring bacteria are able to form a protective biofilm on the leaf surface that prevents the penetration of disinfectants and subsequent lysis of the bacterial cells (LeChevallier *et al.*, 1988). This would imply a less efficient surface disinfection in case of naturally infected plants. On the other hand, biofilms are only (partially) protective against very mild disinfectants like chlorine, but are likely much less, or even not protective against 70% ethanol. Moreover, from the tissue cross sections tested with fluorescent microscopy it was evidenced that *S*. Dublin was present both inside and outside the plant, which suggests the *S.* Dublin CFUs found after surface disinfection were indeed endophytically present in the lettuce plant tissue.

The invasion process observed in this research was similar to the invasion of Barley by *S.* Typhimurium (Kutter *et al.*, 2006). It was suggested that the invasion process of *S.* Typhimurium to colonize plants is similar to that of plant pathogens (Kutter *et al.*, 2006), exemplified by a three-phase process of *Ralstonia solanacearum* infecting hydroponically grown tomato plants (Vasse *et al.*, 1995). First the root surface is colonized, followed by infection of the vascular parenchyma and then invasion of the xylem. This three phase process was also observed in this study. *S.* Dublin was able to colonize the lettuce plant endophytically and epiphytically, both under axenic growing conditions and in manure-

amended soil. Epiphytic movement of *Salmonella* cells from the soil or medium to the aerial portions of the plant via capillary forces was not prevented to retain a non-disturbed colonization and plant-microbe interaction. *S*. Dublin likely first colonized the root surface reaching a high density of bacterial cells around naturally present openings or wounds. This is in line with demonstrated bacterial growth and rhizosphere colonization stimulated by root exudates (Bazin *et al.*, 1990; Cheng *et al.*, 1996) and the observed biofilm formation on the lettuce cross-sections described in this paper. Subsequently, invasion likely occurred via wounds that allowed the bacteria to colonize the roots intercellularly (Cooley *et al.*, 2003; Solomon *et al.*, 2002), but also via intercellular spaces between epidermal cells. Indeed, *S.* Dublin was found in the parenchyma tissue and inside the pericycle, attached to, and inside, the vascular system.

Typically, the stems of sterile grown plants appeared constricted at the root-stem transition point several days after inoculation of the roots with *S.* Dublin. Lettuce may have responded in a hypersensitive manner to the presence of *S*. Dublin. This may have led to reduced nutrient flow, leaf yellowing and finally plant death, indicating that *S.* Dublin might be pathogenic to lettuce under these conditions. A critical point would be that the cell density used for inoculation was rather high with the soil experiments. Indeed, 10^7 cells per g soil are not often found under natural conditions. However, the level of inoculum was applied as a worse case scenario to provide insight in the colonization efficiency of *S.* Dublin on soil-grown plants. To what extent lettuce is still colonized at lower *S.* Dublin cell densities, needs yet to be determined.

In view of the symptoms on plants inoculated with *S*. Dublin, inoculated plants apparently reacted physiologically to colonization by this human pathogen. We also demonstrated, for the first time by cDNA-AFLP analysis, that plant genes were differentially expressed between *S*. Dublin-inoculated and non-inoculated plants. An increase in expression of pathogenicity related genes was observed, which suggest a similar response of lettuce to colonization by *S.* Dublin as by plant pathogenic bacteria. The expression profiles of at least nine genes were strongly associated with the colonization of lettuce by *S*. Dublin. Next to four genes DAD1 (Nakashima *et al.*, 1993), PR1 (Coquoz *et al.*, 1995), PR4 (Ruperti *et al.*, 2002) and PR5 (Uknes *et al.*, 1992) that are known to be related to plant stress, five other genes were obtained from cDNA-AFLP that had differential profiles between colonized and non-colonized plants, namely NAM-like protein gene (Duval *et al.*, 2002), OEE3 gene (Sugihara *et al.*, 2000), PR1-gene (Coquoz *et al.*, 1995), Sec1-family transport protein gene (Aronov and Gerst, 2004), Sec6 transport protein gene (Yeaman *et al.*, 2004), and bHLH016 transcription factor gene (Heim *et al.*, 2003).

The NAM-like protein is involved in shoot development and leaf formation of *Petunia* (Duval *et al.*, 2002). In line with this, plant growth was stunted when colonized with *S.* Dublin and the gene expression was reduced in time compared to healthy plants. For bHLH transcription factor protein genes, the *Arabidopsis* genome encodes for at least 150 putative bHLH class transcription factors, of which many play key roles in phytochrome signal transduction (Heim *et al.*, 2003). These transcription factors are suggested to primarily act more as negative regulators than positive regulators of the phytochrome signalling (Duek and Fankhauser, 2005). Moreover, these bHLH proteins are found to interact specifically with phytochromes. For example, phytochrome-interacting factor 3 (PIF3) mainly acts as a negative regulator in the phytochrome B pathway, but as a positive regulator of anthocyanin and chlorophyll accumulation (Duek and Fankhauser, 2005). The expression profile of the bHLH016 gene identified in this study showed a decrease in time for the colonized plants, but an increase in time for non-inoculated plants. This difference can explain the development of symptoms such as leaf yellowing, implying a reduction of chlorophyll production, which is in line with the expression profile. In addition, yellow leaves also lead to less phytochrome translocation, which is induced by negative regulation of the bHLH proteins.

The Oxygen-evolving enhancer protein 3-2 chloroplast precursor (OEE3) is one of the three OEEs (OEE1, OEE2, OEE3), which are nuclear-encoded chloroplast proteins that are bound to photosystem II (PSII) (Sugihara *et al.*, 2000). Reduction of the gene expression has a direct influence on photosystem II, leading to a reduction of photosynthesis (Sugihara *et al.*, 2000). This might be a secondary effect of colonization by *S*. Dublin, since the bacteria are thought to narrow the stem and herewith reducing the nutrient flow dramatically. The expression profile of the PR1 gene is similar to the previously published expression profile of the PR1 gene of *Medicago* when colonized with *Salmonella* (Dong *et al.*, 2003), which implies a salicylic acid directed defence mechanism of the plant upon colonization with *Salmonella* serovars. These results would suggest a pathogenicity related response of lettuce to colonization by *S.* Dublin.

Interestingly, the expression of genes (sec1 and sec6) involved in the regulation and formation of the actin cytoskeleton (Aronov and Gerst, 2004; Yeaman *et al.*, 2004) was strongly affected during colonization. Inhibition of SNARE (soluble NSF attachment protein receptor) regulatory proteins consequently block mRNA transport by depolarization of the actin cytoskeleton, which will eventually lead to cell death. This might explain the fact that leaf yellowing (and eventually plant death) was observed from the colonized plants. The onset of the secretory block is thought to be activated by a specific signal that influences the actin regulatory machinery. The nature of this signal is not yet known. However, taking into

account the results described in this paper (a strong reduction of the sec1 and sec6 gene expression in time, in contrast to the healthy plants) it might be that the signal is activated by certain bacteria during colonization of the host plant. Since *Salmonella* serovars are known to change / disrupt the actin cytoskeleton prior to invasion of mammalian cells, it might very well be that specific secretory proteins of *Salmonella* serovars are responsible for the blocking of the SNARE regulatory proteins of lettuce. According to this hypothesis the expression profiles of these genes might be related to a more specific than general response of lettuce upon colonization by *S.* Dublin. In that case these genes would be designated as potential marker genes, which is especially of great interest with respect to food safety. This theory should be investigated further to better understand the molecular interaction between lettuce and *Salmonella* serovars during colonization and to clearly identify the presence of such marker genes.

In conclusion, previous studies postulated *Pseudomonas auruginosa* and *Staphylococcus aureus* to be plant pathogenic (Plotnikova *et al.*, 2000; Prithiviraj *et al.*, 2005). Whether *S.* Dublin could also be designated as a pathogen for lettuce cv Tamburo is not fully validated. Under sterile growing conditions symptoms (leaf yellowing, stunting) were observed, whereas no symptoms could be observed on lettuce grown in soil. This might indicate that lettuce Tamburo is susceptible for *S*. Dublin. However, this does not mean that all lettuce cultivars would be equally susceptible to *S.* Dublin, nor that all strains of *S. enterica* would equally efficient at colonizing lettuce. A lettuce cultivar - *Salmonella* strain interaction study would be very interesting and valuable for agriculture and society, in order to reduce, or even prevent the risk of disease outbreaks related to the consumption of fresh produce.

Acknowledgements

This research was supported by the Horticultural Product Board (Produktschap Tuinbouw), the Netherlands. The authors would like to thank Dr. H. Aarts from RIKILT, The Netherlands for providing *S.* Dublin, and Mr M. Raats from Nickerson-Zwaan BV, The Netherlands for providing the lettuce seeds.

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Chapter 5

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

Submitted for publication

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

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Summary

This research describes the differential interaction of human pathogenic *Salmonella* Typhimurium, *S.* Enteritidis, *S*. Dublin, *S.* Newport and *S.* Montevideo with three commercially available lettuce cultivars (Cancan, Nelly, Tamburo). Plant-microbe factors that influence the colonization efficiency were also investigated. The prevalence and degree of endophytic colonization of axenically grown lettuce by the *Salmonella* serovars revealed a significant serovar-cultivar interaction for the degree of colonization (*Salmonella* CFUs / g leaf), but not for the prevalence. The serovars *S.* Typhimurium, *S.* Enteritidis and *S*. Dublin were able to colonize soil-grown lettuce epiphytically, but only S. Dublin was able to colonize the plants also endophytically. The number of *Salmonella* CFU / g of lettuce was negatively correlated to the species richness of the surface sterilized lettuce cultivars. A negative trend was observed for Cancan and Nelly, but not for cultivar Tamburo. In microcapillary tubes *Salmonella* serovars actively moved towards root exudates of lettuce cultivar Tamburo, as visualized by coloration of the metabolic marker tetrazolium; other cultivars were not included in this test. Subsequent micro-array analysis identified genes of *S.* Typhimurium that were activated by the root exudates of cultivar Tamburo. These genes pointed towards a relation with an amino acid source, sugar-like carbon source or pathogenicity factor. These results suggest that the plant-microbe interaction is not a process of chance, but dependent on different factors that may influence the colonization of lettuce cultivars by *Salmonella* serovars.

Introduction

Salmonella sp. are some of the most commonly known bacterial pathogen to cause human illness. Often the disease is associated with the consumption of contaminated foods like pork or poultry meat, eggs or egg products. Since recently, many human pathogenic organisms have been recognized to exist on plant root or leaf surfaces (Brandl, 2006; Lyytikianen *et al.*, 2004), and even inside plant tissues (Kutter *et al.*, 2006; Rosenblueth and Martinez-Romero, 2006). For example, outbreaks of Salmonellosis have increasingly been traced back to contaminated fresh produce (Viswanatan 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 (Natvig *et al.*, 2002; Viswanatan and Kaur, 2001). Both manure and irrigation water contribute significantly to the spread of human pathogens onto fields and the crops growing there (Natvig *et al.*, 2002; Islam *et al.*, 2004; Solomon *et al.*, 2002). To reduce the risk of food-borne illness caused by pathogens on fresh produce, much attention has been given to post-harvest surface disinfection methodologies. Yet, in recent years it became evident that certain human pathogens are not only able to attach to and proliferate on the surface of plant tissues (Solomon *et al.*, 2002; Zenkteler *et al.*, 1997), but can also occur inside plant tissues. For example, the opportunistic human pathogen *Pseudomonas aeruginosa* PA14 was found on *Arabidopsis thaliana* attached to the leaf surface, congregated at the stomata or wounds, and inside the leaves in intercellular spaces (Plotnikova *et al.*, 2000). In addition, gfp-tagged strains of *Salmonella* colonized the interior of tomato plants when grown hydroponically (Guo *et al.*, 2001; Guo *et al.*, 2002) and various *Salmonella* 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.* Typhimurium colonized carrots and radishes which were grown on a field treated with contaminated composted manure or irrigation water (Islam *et al.*, 2004). Just recently, *S.* 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).
Besides the ability of human pathogens to colonize plants, plant defense responses upon colonization by these pathogens have been studied recently. The human pathogenic *Staphylococcus aureus* was able to infect *A. thaliana*, while mutants that contained disrupted genes involved in animal pathogenesis were attenuated in their ability to infect *A. thaliana* (Prithiviraj *et al.*, 2005). Colonization of *Medicago truncatula* by *S.* Typhimurium resulted in the induction of salicylic acid –dependent and –independent plant defenses (Iniguez *et al.*,

2005). The induction of salicylic acid plant defense pathway (SAR) was correlated to the expression of bacterial genes for TTSS-SPI effector proteins, whereas the presence of flagella only induced the SA-independent plant defense (ISR). A recent study involved the molecular response of axenically grown lettuce to colonization by *S.* Dublin, from which a differential expression was indicated of various virulence and pathogenicity-related genes of lettuce, over time (Klerks *et al.*, accepted).

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. Yet, only few studies have been described investigating the conditions required for plant colonization by these pathogens (Rosenblueth and Martinez-Romero, 2006; Toth *et al.*, 2006). Concerning the bacterial genes that are required for attachment to plant roots, several genes have been identified to be crucial for attachment (Barak *et al.*, 2005). Whether attachment only takes place by chance or Salmonellae are actively moving towards plant roots by chemotaxis prior to attachment is not yet known. Moreover, differences in plant cultivar susceptibility might be present, irrespective of the passive or active movement of *Salmonella* serovars to the plant roots. With respect to colonization efficiency differences between *Salmonella* serovars might be expected. Until now no studies have been described concerning differences in cultivar susceptibility or differences between *Salmonella* serovars with respect to colonization efficiency of lettuce plants.

This research describes plant and microbial factors that influence the colonization efficiency of human pathogenic *Salmonella* serovars in association with lettuce. The effect of differences in cultivars and *Salmonella* serovars on colonization efficiency were studied with respect to prevalence and degree of colonization. The role of the 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 *Salmonella* CFU / g lettuce. Finally, the contribution of root exudates to colonization efficiency of lettuce by *Salmonella* 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 *Salmonella enterica, subspecies enterica,* serovar Dublin, serovar Typhimurium, serovar Enteritidis, serovar Newport and 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., UK) and were increased by overnight enrichment at 37°C in buffered peptone water (BPW).

Seeds of 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 hour and stored.

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

To determine if *Salmonella* serovars are able to colonize lettuce seedlings via the roots under semi-natural conditions, lettuce seeds (cultivar Tamburo) were planted on 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 mg / kg and 22400 mg / 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 / kg and 8167 mg / kg, 220 mg / kg ammonium, 8.14 mg / 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 1g of each substrate using real-time PCR analysis (Klerks *et al.*, 2004). The manure was first inoculated with either *S.* Typhimurium, *S.* Enteritidis or *S.* Dublin (10^8 CFUs / g fresh weight) and mixed thoroughly before addition to soil at a ratio of 1:10 fresh weight. The final *Salmonella* cell density was 10^7 CFU / g mixture. In total 74 pots of 50 ml of each contaminated soil-manure mixture were prepared per *Salmonella* serovar. The negative control pots (74) consisted of non-*Salmonella*-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 hours 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, the pellet was re-suspended in 100µl of BPW and 40µl 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.*, in

press). Of each suspension with ground plant material 40µl was plated on Hektoen enteric agar, in duplicate.

Differential colonization of lettuce cultivars with Salmonella enterica serovars

To evaluate whether the endophytic colonization efficiency of lettuce by *S. enterica* is dependent on the lettuce cultivar or the *Salmonella* serovar, three lettuce cultivars were tested with five *Salmonella* serovars for the degree of endophytic colonization. First, sterile seeds of three cultivars Cancan, Tamburo and Nelly, were sprouted in sterile 0.5 % Hoaglands water agar (Sigma Aldrich) in 15 ml glass tubes and incubated for 2 weeks at 21°C with light/dark intervals of 12 hrs 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.* Typhimurium, *S.* Enteritidis, *S.* Dublin, *S.* Montevideo and *S.* Newport), resulting in nine replicates per combination in three blocks. A Fresh BPW culture of each *Salmonella* serovar was 100-fold diluted in sterile Hoagland's' solution and 10 µl (10^7 CFU/ml) 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 sec in 70% ethanol and rinsing twice with sterile water (Klerks *et al.*, accepted). Then, leaf tissue was thoroughly ground in cold BPW. For downstream molecular analyses 100µl of suspension was used to extract DNA. A dilution series was prepared (non-diluted, 20x and 200x diluted) from the ground tissue suspension and 40µl of each dilution series was plated in duplicate on *Salmonella*-selective Hektoen enteric agar and incubated overnight at 37°C. The colonies grown from the dilution series were counted and a random selection of *Salmonella*-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. DNA was pelleted from these enrichment cultures by centrifuging 500µl at 6000g for 5 min. Next, the DNA was extracted from the pellet according to the protocol of the microbiological DNA extraction kit (Mobio). The purified DNA was eluted in 100µl of elution buffer and stored at -20ºC until further use.

Detection and identification of Salmonella enterica

Salmonella 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 100µl of suspension which was added to 400µl of lysis buffer supplied with the kit. Further treatment was according to the supplied protocol of the DNA

extraction kit (Westburg, Qiagen). The purified DNA was eluted in 200µl 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.

Relation between Salmonella colonization and the endophytic microbial community

To investigate a possible relation between endophytic colonization by *S. enterica* and endophytic microbial communities, the diversity index, Shannon index (H) (Shannon and Weaver, 1963), and the species richness of 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 *Salmonella* populations and one *Salmonella*-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 *Salmonella* serovar) were first subjected to 16S rDNA PCR using bacterial ribosomal genome primers that excluded chloroplast amplification (Rochelle *et al.*, 1992; Chelius and Triplett, 2000). The PCR reaction mix (final vol. 24 µl) consisted of 200 µM dNTP, 3.75 mM MgCl2, 1x Stoffel buffer (Applied Biosystems, Foster City, USA), 0.4 µM primer799F(Chelius and Triplett, 2000), 0.4 µM primer1492R(Rochelle *et al.*, 1992), 0.05% BSA and 0.1 U Amplitaq Stoffel polymerase. To each reaction tube 1µl of 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 sec at 94°C, 40 sec at 53°C and 40 sec at 72°C. The reaction was stopped by 7 min incubation at 72°C.

The primary PCR was followed by a nested 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 nested PCR reaction mix (total volume of 49µl) consisted of 200 µM dNTP, 1x SuperTaq buffer (Applied Biosystems, Foster City, USA), 0.4 µM primerU968, 0.4 µM primer R1378, and 1 U SuperTaq polymerase. To each reaction mix 1µl of primary PCR product was added and 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 reaction was stopped by 10 min incubation at 72°C followed by 5 min incubation at 10°C.

Of each nested PCR product 20µl was mixed with 10µl of loading buffer and applied to the gradient gel. The 6% (wt/vol.) polyacrylamide gels in 0.53 TAE buffer (20 mM Tris-acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM disodium EDTA) contained a linear denaturing gradient of 45 to 65% of urea and formamide. The gels were run for 15 h at 60°C and 100V. 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 UV light (Biozym, Landgraaf, the Netherlands).

Response of Salmonella serovars to root exudates of lettuce Tamburo

To determine if root exudates affect the movement *Salmonella* 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 separate the organic-soluble compounds from water-soluble compounds, 1ml 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) was transferred to a new tube, 1 ml of fresh ethyl-acetate was added and mixed thoroughly. After centrifugation the water-phase (WP) was transferred to a new collection tube and stored at -20ºC until further use. The final exudate concentration was similar to 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 50µl, diameter of 1mm) were filled with 0.2% of Hoagland's agar, including 0.5% of the metabolism marker 2, 3, 5-triphenyl tetrazolium chloride (TTC) to measure bacterial movement. The WP of three lettuce plants (50 µl of WP per plant) were each tested in duplicate for chemotaxis of *S.* Typhimurium, *S.* Enteritidis, *S.* Dublin and water. Of each serovar 50µl of 10^7 CFU/ml 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 37ºC prior to observation of chemotaxis by color transition inside the capillaries.

Gene expression of Salmonella *Typhimurium in presence of root exudates*

To investigate the molecular response of *Salmonella* serovars to the presence of root exudates, gene expression of *S.* Typhimurium exposed to root exudates was analyzed over time. First, fresh enriched bacterial cultures of *S.* Typhimurium were prepared, centrifuged, washed twice with phosphate buffer and finally re-suspended in phosphate buffer. Four tubes were prepared containing 900µl 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 100µl of each bacterial suspension was collected and immediately transferred to 350µl of lysis buffer of the RNeasy RNA extraction kit. The time series consisted of 0, 10, 20 min post inoculation. Next, total RNA was extracted from 100µl of the bacterial suspensions using the RNeasy RNA extraction kit (Westburg, Qiagen). RNA was eluted in 100µl of RNase-free water and stored at -80ºC until further use.
From the time series cDNA was prepared to allow gene expression analysis using a thematic micro-array of *S.* Typhimurium virulence, growth and stress-related genes (Hermans *et al.*, 2007). Each sample for cDNA synthesis was subjected to amino-allyl-dUTP labelling for subsequent Cy3 and Cy5 labelling, according to the protocol described by Hermans *et al.* (2007). After cDNA synthesis and Cy3 or Cy5 labelling, the cDNA was precipitated according to general sodium acetate / ethanol precipitation. After drying of the pellet the cDNA was re-suspended (in duplicate) in filter sterilized hybridization buffer (0.2% SDS, 5x Denhardt's solution, 5x SSC, 0.5x formamide and 0.25ug 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 *et al.*, 2007), the data was corrected for inter-chip and intra-chip variations and a-specific labeling as described by Hermans *et al.* (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

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

To determine the effect of lettuce cultivar or *Salmonella* serovar on the prevalence of *Salmonella* CFU on/in lettuce seedlings a non-parametric test (Kruskall-wallis) was performed. The interaction was determined by a Chi-square test based on 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 / 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 4 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 *Salmonella* serovars (log CFU / g), using Pearson correlation analysis (SPSS). In addition, the log CFU / g was regressed on both diversity parameters.

To determine the significance of the root exudate treatment relative to the controls, chi-square analysis was performed on 54 capillary tubes which were positive or negative for chemotaxis of *Salmonella* 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 the different serovars was also compared with a chi-square test.

Results

Colonization by Salmonella *serovars of lettuce Tamburo grown on manure-amended soil*

Of the 75 seeds planted per *Salmonella* 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.* Dublin, 8 out of 48 with *S*. Enteritidis and 14 out of 53 with *S*. Typhimurium. The number of emerged plants was significantly different between the non-inoculated plants and the *Salmonella*-inoculated plants (X^2=9.231; P=0.026). The number of colonized plants did not differ significantly among serovars (X^2=2.056; P=0.358). The control plants were all negative for *S. enterica*. For *S*. 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*. Enteritidis or *S*. Typhimurium. Taking the prevalence and degree of colonization per positive plant into account, the total endophytic colonization from inoculated manure-amended soil was only 164 CFU / g lettuce for *S*. 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, six weeks after planting of lettuce seeds.*

	Manure-amended soil	**Hoagland's agar**
	Mean CFU / g	Mean CFU / g
*S.*Dublin	164	40800
S Typhimurium	0	18739
S. Enteritidis	0	8506

Endophytic colonization of lettuce cultivars by Salmonella *serovars on Hoagland's agar*

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

Univariate analysis of variance indicated that there was a significant interaction between serovar and cultivar with respect to the degree of endophytic colonization (CFU / g leaf) (p=0.047). This suggested a difference in colonization pattern of a specific lettuce cultivar by the five *Salmonella* serovars, in particular S. Typhimurium colonized cultivar Nelly more easily than the other cultivars (Figure 1). The overall lettuce cultivar effect on internal colonization (CFU/g leaf) was not significant (p=0.116), while the *Salmonella* colonization was significantly affected by *Salmonella* serovar (p=0.004) (Figure 2).

Taking both the prevalence and degree of colonization per positive plant into account, the total endophytic colonization from inoculated manure-amended soil was highest for *S*. Dublin and much lower for *S*. Typhimurium and *S*. Enteritidis, similar to the overall colonization in manure amended soil, although the level of colonization was much higher in Hoagland's agar than in soil (Table 1).

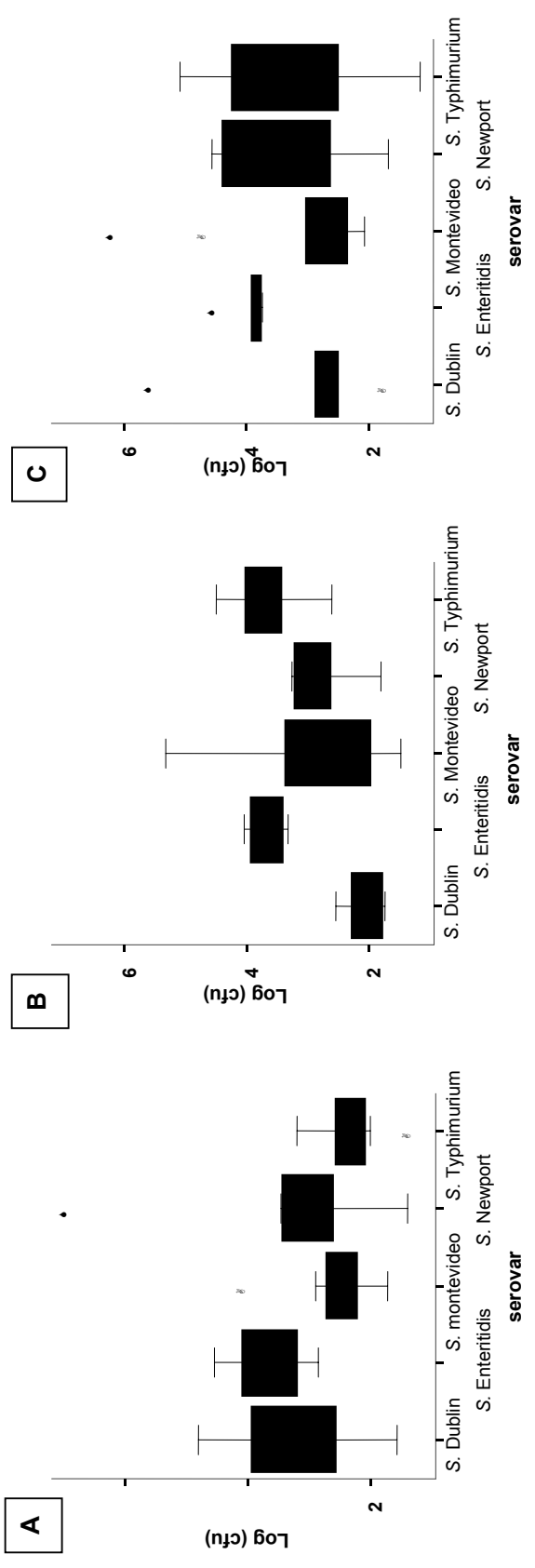

Figure 1. *Box plot of the degree of colonization per serovar* Salmonella *Dublin,* S. *Enteritidis,* S. *Montevideo,* S. *Newport and* S. *Typhimurium for each cultivar Cancan (A), Nelly (B) and Tamburo (C) separately.*

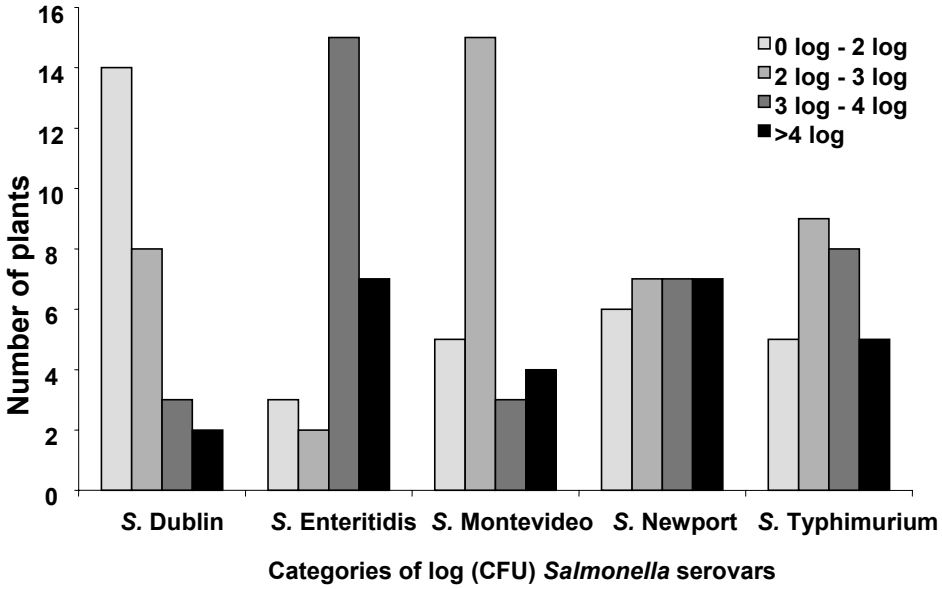

Figure 2. *Number of lettuce seedlings in different classes of degree of* Salmonella*-colonization for each* Salmonella *serovar tested.*

Endophytic microbial communities of Salmonella*-colonized lettuce cultivars*

Correlation analysis of the endophytic species richness based on number of DGGE bands versus the degree of endophytic colonization (log *Salmonella* CFU / 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*), based on the number and relative intensity of the bands on a sample lane, versus the degree of endophytic colonization (log Salmonella CFU / gr) was not significant (r= -0.276; p= 0.066).

Response of Salmonella *to lettuce root exudates*

The root exudates of three Tamburo plants resulted in metabolic activity (red coloring) inside the micro-capillaries of *S*. Typhimurium (8 out of 12 positive), S. Dublin (5 out of 12) and S. enteritidis (0 out of 12) (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 Chi square 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-square=8.56; p=0.05). There

were also significant differences among the serovars with respect to number of positive reactions in response to the root exudates (Chi-square=11.8; p =0.01). There was no differences between *S.* Typhimurium and *S.* Dublin (Chi-square=1.5; p=0.53), but there were differences between *S.* Dublin and *S.* Enteritidis: (Chi-square=6.3; p=0.05) and between *S.* Typhimurium and *S.* Enteritidis (Chi-square=12; p=0.01).

Gene-expression of Salmonella *Typhimurium in response to lettuce root exudates*

To test the response of *Salmonella* serovars upon exposure to root exudates, micro-array analysis was performed on the time series of *S*. Typhimurium (0, 10 and 20 min post inoculation) of each treatment. After normalization (according to Hermans *et al.* 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 towards a relation with a sugar-like carbon source.

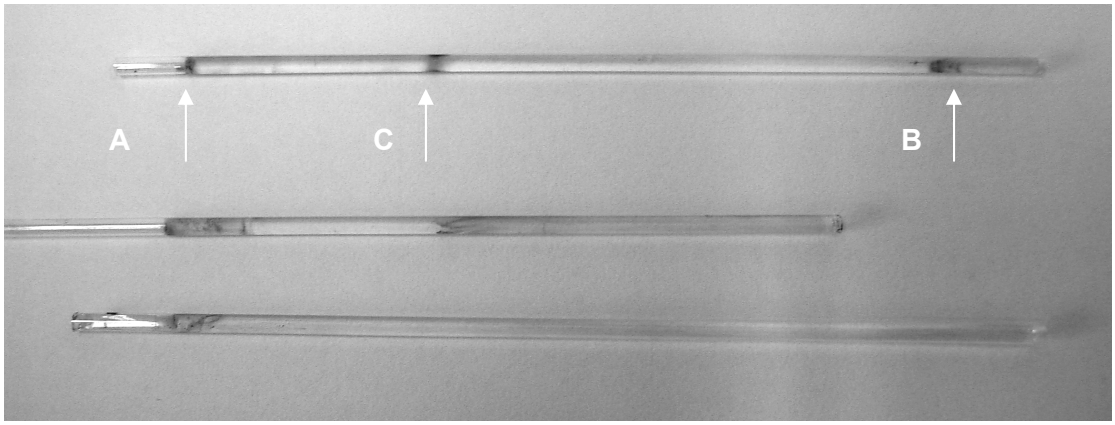

Figure 3. *Chemotaxis of* Salmonella *serovars in microcapillary tubes with root exudates or control solutions traced by reaction with 2, 3, 5-triphenyl tetrazolium chloride. The left side of a microcapillary tube was placed in a suspension of* Salmonella *(or water as control) present in a 0.5 ml eppendorf 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 towards 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.*

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. The level of normalized gene-expression is indicated for each gene per treatment in time. The increase (white) of normalized gen-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).*

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 genes related to a sugar-like carbon source were *Ots*A (trehalose-6-phosphate synthase), which utilizes glucose-6-phosphate as substrate (Giæver *et al.*, 1988), *Uhp*C (hexose phosphate utilization protein), which is a sensor for external glucose-6-phosphate)

(Schwöppe *et al.*, 2003), *Met*E (methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase), a vitamin B12-independent enzyme (Urbanowski and Stauffer, 1989), *Dsr*A (putative anti-silencer RNA), a regulator of transcription to express *Rcs*A promoter, which on its turn is responsible for capsular polysaccharide synthesis (Sledjeski and Gottesman, 1995), *Rse*A (sigma-E factor regulatory protein), which is involved in 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).

Discussion

Several human pathogenic organisms have been recognized to exist on plant root or leaf surfaces (Brandl, 2006; Lyytikianen *et al.*, 2004), and even inside plant roots (Kutter *et al.*, 2006, Rosenblueth and Martinez-Romero, 2006). However, only few studies have investigated the physiological or molecular interaction between human pathogenic bacteria and plants (Iniguez *et al.*, 2005; Klerks *et al.*, accepted; Kutter *et al.*, 2006; Plotnikova *et al.*, 2000; Prithiviraj *et al.*, 2005). Even fewer studies have been described concerning the conditions required for plant colonization by these pathogens (Toth *et al.*, 2006).

This research presents plant and microbial factors that influence the (endophytic) colonization efficiency of human pathogenic *Salmonella* serovars in association with lettuce. Next to pathogenicity genes that are crucial for root attachment and subsequent colonization (Barak *et al.*, 2005), also genes for general metabolism appeared to be of importance for *Salmonella* serovars to actively move to the plant roots. In addition, a differential *Salmonella* serovar-dependent host susceptibility was observed, suggesting the presence of host-adapted serovars but also more resistant cultivars. Finally the natural endophytic microflora was observed to contribute to the level of susceptibility of the lettuce cultivar for *Salmonella* serovars. The active movement of *Salmonella* 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 was visualized using a metabolism marker (TTC) which also suggests the presence of an organic compound in the lettuce root exudates used as carbon source by the *Salmonella* serovars. In another study it was found that pathogenicity-related genes of *Salmonella* serovars were crucial for attachment to plant roots (Barak *et al.*, 2005).

Up to now it was not known to what extent pathogenicity-related genes would also play a role in chemotaxis. In our micro-array analysis most genes that were differentially expressed were nutrition-related genes associated with the presence of chemotaxis-inducing root exudates. Fewer genes appeared to be related to pathogenicity of *Salmonella*. These data confirmed the macroscopic observations in the chemotaxis experiment. Chemotaxis of *Salmonella* serovars to sugar suspensions was already described in the late 70s (Melton *et al.*, 1978) and it is also well known that root exudates contain various (mono)-saccharides like fructose, glucose, etc (Neumann and Römheld, 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). Thus, sugar-like compounds in the root exudates can be responsible for the active movement of *Salmonella* serovars to the roots. In addition, amino acids could 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 up-regulated (Urbanowski and Stauffer, 1989). Further analysis and identification of these organic compounds by HPLC 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 *Salmonella* serovars to the roots. In combination with gene-expression profile determination by RT-PCR this eventually might lead to identify marker genes for chemotaxis.

Several genes that were differentially expressed in our micro-array were associated with pathogenicity and could be involved in attachment to host cells. 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 i.e. secretory proteins. The observed induction of *Dsr*A might also imply 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). These findings suggest that *Salmonella* serovars are triggered by the root exudates to actively move towards the roots and are also conditioned for attachment to the plant root surface.

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). In soil the Salmonellae have to compete with these environmental bacteria to establish in the rhizosphere (Cooley *et al.*, 2003; Ibekwe *et al.*, 2006; Gagliardi and Karns, 2000), leading to lower *Salmonella* cell densities close to the roots. This suggests that the colonization efficiency is strongly dependent on accessibility of the plant roots by the Salmonella serovars.

Salmonella serovars colonized lettuce endophytically as was shown before (Cooley *et al.*, 2003; Klerks *et al.*, accepted). However, we showed for the first time a differential interaction between *Salmonella* serovar and plant cultivar, besides serovar-dependent host susceptibility with respect to degree of colonization. This result points to differences in susceptibility of the cultivars, but also differences between the *Salmonella* serovars with respect to colonization of lettuce seedlings. Next to being exposed to plant defenses, *Salmonella* serovars also have to compete with the plant endophytic microflora for a certain niche with carbon sources (Leveau and Lindow, 2000). The species richness of the endophytic microbial community of lettuce cultivars was negatively correlated to the number of endophytic *Salmonella* CFUs / 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 *Salmonella*, or at least limiting the endophytic colonization by *Salmonella* serovars compared to cultivar Tamburo. Of course, measuring the complexity of the microbial community is inherently biased since only the most abundant species (maximum of appr. 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, 1993). Thus, a less abundant species might have a higher impact on the colonization efficiency of *Salmonella* serovars than the visualized species from DGGE analysis.

In conclusion, from this study it is evident that the *Salmonella* serovars and lettuce cultivars interact significantly at the population and molecular levels of integration with respect to prevalence and degree of infection. Several plant and bacterial factors were identified from the tentative route of infection which *Salmonella* has to counter prior to plant colonization.

Acknowledgements

This research was supported by the Horticultural Product Board (Produktschap Tuinbouw), the Netherlands. The authors would like to thank Dr. H. Aarts and Drs. A. van Hoek from

RIKILT, The Netherlands for providing the *Salmonella* serovars and the Salmonella Micro-arrays, including practical assistance. We would also like to thank Dr. A. Speksnijder for his expertise in DGGE analysis and gel comparisons, Mr. F. Verstappen for his contribution to the preliminary LC-MS and GC-MS analysis, and Dr. A.M. Semenov for his valuable comments in reviewing this manuscript.

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Chapter 6

General discussion

Introduction

Human pathogenic bacteria that reside in the environment have gained major interest due to an increase in produce-associated outbreaks during the last decades (Sivapalasingam, Lyytikianen *et al.,* 2004, Viswanathan and Kaur, 2001). Especially since sanitation procedures during food processing (for example washing with chlorine) are not sufficient in eliminating the associated pathogens, a more stringent microbial monitoring of the vegetable chain from feed to fork is required to reduce health risks (Brooks *et al.,* 2001).

Recently, prevention of contamination has been stressed even more by the finding that some pathogens are able to colonize plant tissues internally (Kutter *et al.,* 2006). To maintain safe food, the chance of contamination of produce in the production chain needs to be reduced. 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. In that respect, insight in the type(s) of interaction is required with respect to the plant response to colonization by human pathogenic bacteria during plant growth. To ensure pathogen-free produce for consumption robust detection methodologies need to be implemented in pathogen monitoring procedures of the vegetable chain that allow reliable quantification of pathogens in different environmental substrates.

Some fundamental questions need to be answered before such preventive strategies can be defined. To which extent are differences present between plant cultivars or bacterial strains with respect to colonization efficiency? Do human pathogenic bacteria become associated with plants in a passive manner, or are the bacteria actively moving towards plant roots due to chemotaxis? Which bacterial genes are involved during the various stages of colonization? In order to prevent the colonization of vegetables by human pathogens there is an urgent need to know all relevant factors affecting the colonization efficiency.

Additive value of molecular methods in food diagnostics

In recent years the use of molecular tools to detect pathogens has increased substantially. However, certified procedures for detection of pathogens like *Salmonella enterica* and *Escherichia coli* O157:H7 in food are still based on standardized methods that are dependent on plating techniques combined with biochemical serotyping (ISO 6579/2002 and ISO 16654/2001, respectively). At present, food samples are randomly selected for routine diagnosis and analyzed independently from the production line. Often the products have

already been transported, or even worse, consumed by the end-users days before the results of the microbial analyses are available. This is especially the case for spoilage-sensitive products like (pre-cut) produce and fruits. Consumers and governmental agencies demand high quality food that is certified to be free of pathogens.

As a result of the availability of molecular tools like real-time PCR (Holland *et al*., 1991), much emphasis is put on on-line testing of food samples. These molecular methods appear promising for faster analysis of food samples during routine diagnostic screenings in a high throughput setting due to the reduction in assay time from 5 days (standardized plating methods) down to a maximum of 2 days. Most of these PCR-based methods are also supposed to enable quantification of initial amount of target organisms in a substrate sample. Quantification is essential to determine the bacterial load of a food sample. For each pathogen a threshold is determined at which a food sample is still acceptable for further processing. For example, *Staphylococcus aureus* is not acceptable in a produce sample if 100 colony forming units per gram, or more, are present. In meat this threshold is set at 500 CFU per gram for *S. aureus*, as well as for *E. coli*. Therefore, quantification is vital for the food processing industry to ensure high quality and safe food.

In this thesis, several quantitative methods to detect *S. enterica* or *E. coli* O157:H7 were compared with respect to sensitivity, precision and accuracy (Chapter 2, Klerks *et al*., 2004). As expected, the precision and accuracy decreased dramatically when the amount of target DNA was close to the detection limit. This is inherent to amplification methods. Since the obtained sensitivity of the methods was 10 fg of bacterial genomic DNA (equal to 2 CFUs / reaction which is close to the theoretical detection limit of 1 CFU), quantitative analysis was only valid from 2 log higher amounts of DNA. However, the time reduction is of primary interest for the processing industry, especially since most of the food samples will not contain the pathogen (~99%) (Struik Foods Belgium BV, personal communication). The samples can first be screened using a PCR test for qualitative detection (with a reliable detection limit of 2 CFU / reaction) in combination with a-selective enrichment. Since a large number of the tested food samples will likely test negative for presence of the pathogen, within one day these pathogen-free food samples can directly be processed further (accounting for ~99% of all tested samples). As a consequence, the required time during quality testing of the food samples is reduced enormously. Only the few samples that were determined positive for the pathogens need further evaluation according to the standardized methods (which can take up to 5 days).
With this approach, discrimination between false negatives and true negatives is of high importance but is not enabled by most available PCR methods. This can be countered by

introduction of an internal control for amplification. In contrast to other amplification control approaches, during this thesis research an internal control was developed that can be used irrespective of the substrate or target organism to be detected (Chapter 2, Klerks *et al*., 2004). This control is based on the *gfp*-coding sequence (Prasher *et al.,* 1992), derived from an organism not found in environmental substrates associated with on-land food production. The precision and accuracy of the methods for detection of *S. enterica* or *E. coli* O157:H7 retained, while the sensitivity is reduced only 10-fold. Moreover, the internal control described in this thesis enables verification of the reliability of quantification of the target that is tested in real-time PCR detection methods, in the sense that changes in amplification efficiency of the internal control are presented as a shift in Ct-value during PCR. If this Ct-value is statistically different from the no template control samples (but with internal control), based on the PCR results the target quantification is not representative to the amount of target present in the initial sample. Application of this internal control enables the evaluation of reliability of quantification for each separate amplification reaction for detection of *S. enterica* or *E. coli* O157:H7 (Chapter 2, Klerks *et al*., 2004). Currently this approach is also used in other real-time PCR methods to validate the amplification efficiency (Zijlstra and Van Hoof, 2006), but mainly in those cases where the presence of inhibiting agents can be expected.

As mentioned earlier, the threshold for reliable quantification is 100-fold higher than the detection limit of the methods described. Moreover, prior to PCR amplification several dilution steps are introduced during DNA extraction procedures, theoretically resulting in a detection threshold of 8000 CFU / g sample. Eliminating the dilution steps from the procedure, theoretically a sensitivity of 80 CFU /g sample should be possible. This would favor these methods substantially over currently used plating methods. Up to now most attempts to omit the dilution steps have been hampered by the concurrent increase of inhibiting agents or excessive DNA, which reduce the efficiency of either DNA extraction or amplification. As a consequence, the applicability of most real-time PCR methods is not fully used in routine diagnosis, since these methods do not yet meet the requirement of enabling quantification of low levels of pathogens.

Influence of environmental substrates on quantification of Salmonella enterica

Many DNA extraction procedures are known to have variable extraction efficiencies for different substrates (Lloyd-Jones and Hunter, 2001; Theron and Cloete, 2000) mainly due to the large variation in biochemical composition (Wilson, 1997; Al-Soud and Rådström, 1998).

Extraction efficiencies have a large effect on the reliability of quantification based on molecular techniques like PCR. Remarkably, only few papers compare the extraction efficiency of commercial DNA extraction methods for soil, manure or compost (Zhou *et al*., 1996; Lloyd-Jones and Hunter, 2001). In this thesis, six different DNA extraction methods were compared with respect to DNA extraction efficiency and reliability of quantification of *S. enterica* present in substrate samples being soil, manure or compost (Chapter 3, Klerks *et al*., 2006). Additionally a control method for DNA extraction was developed. The DNA extraction efficiency was assessed by applying a fixed amount of *E. coli* that harbored a plasmid containing the *gfp*-sequence to the substrate prior to DNA extraction. Subsequent PCR analysis also uses this *gfp*-control as amplification control, herewith giving insight in both the DNA extraction efficiency and the amplification efficiency. Using this general internal procedural control (GIPC) approach a sensitivity of at least $2x10^3$ CFU/100 mg of substrate was obtained using DNA extraction followed by Taqman PCR. After enrichment even 1 CFU of *S.* Enteritidis was clearly detected per 100 mg of substrate. By using the GIPC, the presence of inhibitory factors was determined in a similar manner as described in Chapter 2 (Klerks *et al.*, 2004).

From this study it was also evident that, despite the inclusion of a procedural control, quantification errors still occurred when detecting a pathogen like *S.* Enteritidis in environmental substrates. These errors are introduced by the sum of the variance of the methods for extraction and amplification, representing the error in the procedural quantification. Even though a quantification error remained, the application of the GIPC enabled verification of the reliability of quantification for *S. enterica* in environmental substrates compared to methods omitting a procedural control.

The theoretical detection threshold for *S. enterica* after DNA extraction followed by PCR amplification was determined as 8,000 CFU / g (previous paragraph). This means that a reduction of 60% of DNA available for PCR amplification is introduced as a consequence of DNA extraction from environmental substrates (20,000 CFU / g). In order to dilute the inhibiting agents to non-influencing concentrations, the extracted DNA is diluted 10-fold, which was also included in the calculation of the theoretical threshold. This results in a total reduction of 96% of available target DNA for PCR amplification. Despite of the re-occurring problem of PCR inhibiting agents, no such agent has yet been identified chemically. Co-extracted contaminants like humic and fulvic acids are known to cause problems during PCR amplification (Al-Soud and Rådström, 1998; Fortin *et al.,* 2004). Unidentified components different from humic and fulvic acids but commonly present in soil have also been related to PCR inhibition (Watson and Blackwell, 2000). We attempted to identify the inhibiting

components by performing liquid-chromatography mass-spectrometry analysis of DNA extracts from soil and manure types. Unfortunately these preliminary analyses did not lead to differential peaks between inhibiting extracts and non-inhibiting extracts. Despite this outcome, we suspect that application of such chemical analysis approaches will eventually elucidate the agents responsible for the variance and inhibition in DNA extraction and subsequent amplification and will concurrently lead to improvements of the DNA extraction and subsequent amplification methods.

Tentative route of plant infection by human pathogenic Salmonella *serovars*

To develop preventive strategies it is important to elucidate the critical points of plant colonization by the pathogen. Based on previously published data and the research from this thesis we have defined a tentative route of infection of lettuce plants by *Salmonella* serovars. Basically, the *Salmonella* serovars that are applied with the 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 *Salmonella* serovars are triggered by sugar-like root exudates and due to chemotaxis the bacteria move actively towards the roots (Chapter 5, this thesis). The ability of *Salmonella* serovars to produce flagella and highly sensitive sensors for chemotaxis or quorum sensing compounds contributes largely to the rate of its movement (Melton *et al.*, 1978). In close proximity to the roots, the 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 *Salmonella* cells to compete with rhizosphere bacteria for nutrients. In addition, it was postulated that part of the quorum sensing mechanism of *Salmonella* allows it to intercept signals from other bacteria, but in the meanwhile the *Salmonella* cells remain 'invisible' for the surrounding bacterial microflora (Ahmer *et al.*, 2004). 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 (Chapter 4, Klerks *et al*., accepted). However, for biofilm formation attachment to the roots is required. This is primarily dependent on the presence of curli and lipopolysaccharides (Barak *et al.*, 2005). Colonization of the plant roots by bacteria generally depends on the presence of flagella, the O-antigen of lipopolysaccharides (LPS), the growth rate and the ability to grow on root exudates (Lugtenberg and Dekkers, 1999).

Up to this point, the route of infection is mainly driven by *Salmonella*, although plant-

associated rhizosphere bacteria and root exudates also play an important role prior to intimate contact between *Salmonella* cells and the plant host. Upon attachment, a biofilm may be formed and as soon as the population densities have reached a level at which plant defenses can be overcome, the production of pathogenicity factors may be induced (Miller and Bassler, 2001; Von Bodsman *et al.*, 2003). Quorum sensing-driven secretion of cell wall degrading enzymes could facilitate gaining access to the inner membrane of the plant cells (Miller and Bassler, 2001). Upon cell disruption or presentation of membrane-bound pathogenicity factors like flagella (Iniguez *et al.*, 2005), the plant responds in a hyper sensitive manner with subsequent triggering of ISR. 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 (SAR). To what extent the plant responds by ISR and SAR 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. Interestingly, signalling compounds (*N*-acyl homoserinelactone) of *Serratia liquefaciens* that are involved in quorum sensing also induce components of the innate immune response of tomato plants, like the PR1 protein and chitinase (Hartmann *et al.*, 2004). This might point towards an additional response pattern next to SAR and ISR. Finally, the endophytic pathogenesis follows that of plant pathogens by first infecting the vascular parenchyma and then the invasion of the xylem to allow systemic infection (Chapter 4, Klerks *et al*., accepted).

Unraveling basic interactions

The recent outbreaks of Salmonellosis in association with fresh produce gave rise to the questions whether the plant responds to the presence of, and colonization by *Salmonella*, and if so how and to what extent. Recent research has focused more on the colonization of plants by human pathogenic bacteria (Plotnikova *et al.*, 2000; Prithiviraj *et al.*, 2005; Iniguez *et al.*, 2005). During colonization of roots of *A. thaliana* by *S. aureus*, resistance was mediated by a direct effect of salicylic acid on *S. aureus*, affecting the attachment on the root surface and reducing pathogen virulence (Prithiviraj *et al.*, 2005). Another study revealed that salicylic acid-dependent and -independent defenses were induced in *Medicago truncatula* upon colonization by *S.* Typhimurium (Iniguez *et al.* 2005). However, not only the PR1-gene was expressed upon colonization of lettuce by *Salmonella* serovars in time, but also many other plant defense-related genes, for example reactive oxygen species (ROS)-related genes and other pathogenicity related (PR)-genes (Chapter 4, Klerks *et al*., accepted).
An ISR response was at least triggered by the presence of flagella of *Salmonella* serovars during root colonization and biofilm formation, followed by the induction of the SAR defense

system by the type III effector proteins of *Salmonella* (Iniguez *et al.*, 2005). However, the outer membrane structure of the type III secretion system (needle-like structure) of *Salmonella* is smaller than that of plant pathogens (more pilus-like) and thus not directly effective for penetration and subsequent translocation of effector proteins (Romantschuk *et al.*, 2001). It was hypothesized that, like with plant pathogens, during biofilm formation cell wall degrading enzymes are produced by *Salmonella* serovars to provide access to the plant cell membranes. In line with this hypothesis a cellulase was isolated from *S.* Typhimurium that was able to degrade cellulosic substrates (Yoo *et al.*, 2004). However, recent fluorescent microscopy analysis presented *Salmonella* cells mainly at intercellular spaces between epidermal cells during internalization (Chapter 4, Klerks *et al.,* accepted), herewith suggesting a preference for the intercellular spaces and not the plant cell itself. Thus, a complete description of the cell-cell interaction from attachment to internalization is not yet available.

Further descriptions concerning colonization of *Salmonella* serovars in plants and plant cells are not known. It would be of interest if the infection process of plants is similar to that of mammalian hosts at a cellular level. Evidence of similarity was found based on gene-expression analysis. The secretory protein genes sec1 and sec6 were strongly affected with respect to level of gene-expression in the plants colonized by *Salmonella* (Chapter 4, Klerks *et al*., accepted). These genes are involved in the regulation and formation of the actin cytoskeleton of the cells (Aronov and Gerst, 2004; Yeaman *et al*., 2004), also known as one of the main key features of *Salmonella* pathogenesis during mammalian cell invasion. Also an increase in number of differentially expressed ROS-related genes (part of plant defense system) was observed after colonization. In mammalian cells ROS-related genes also have a defensive function, in which *Salmonella* serovars are able to prevent the co-localization of oxidase (Vasquez-Torres *et al.*, 2000) and nitric oxide synthase (Chakravortty *et al.*, 2002), herewith protecting themselve from reactive oxygen intermediates and reactive nitrogen intermediates. In plants this protecting ability might also play an important role, perhaps in a similar manner as with mammalian cells, to allow further pathogenesis.

It is evident that lettuce responds in a defensive manner if colonized by *Salmonella* serovars, presumably in a similar way as with plant pathogens. Nevertheless, this does only contribute partly to the assumption that the human pathogenic *Salmonella* serovars are also plant pathogenic. When lettuce was grown axenically, symptom development occurred from stunting to leaf yellowing, stem narrowing and eventually plant death (Chapter 4, Klerks *et al*., accepted). However, when lettuce was grown in soil, no physiological response was observed, even though *Salmonella* serovars were present epiphytically and endophytically. It

appears that lettuce is a tolerant host when grown in soil, whereas under semi-sterile conditions lettuce appears to be a susceptible host. This implies that microbial antagonism and competition prevents *Salmonella* serovars to become pathogenic to lettuce plants (no symptom development, reduced epiphytic and endophytic colonization), at least during the seedling stage.

From the specific plant responses upon colonization by *Salmonella* it was hypothesized in this thesis that differences in colonization efficiency and host susceptibility might occur between *Salmonella* serovars and lettuce cultivars (Chapter 5, this thesis). Indeed differential serovar-dependent host susceptibility was observed, which indicated a difference in host adaptation of *Salmonella* serovars, but also the likelihood of existence of (partially) resistant lettuce cultivars. Also the endophytic microflora contributed to the level of susceptibility of the lettuce cultivar for *Salmonella* serovars (Chapter 5, this thesis). From these data it was concluded that the lettuce endophytic microflora was antagonistic to *Salmonella* serovars, although the level of antagonism was dependent on the cultivar. In that sense it can be suggested that the endophytic microbial population contributes (even though to a minor level) to plant defense or differences in susceptibility. Optimization of the plant (endophytic) microflora might therefore result in plants that are most conditioned for resistance to *Salmonella*.

However, colonization of lettuce plants by *Salmonella* serovars was mainly dependent on the level of pathogenicity of *Salmonella* serovars, rather than the defense systems of the plant. Similar to bacterial pathogens in general, the differences in colonization efficiency between the *Salmonella* serovars are assumed to be related to genetic differences, like the presence of host-directed genes and / or due to a more advanced host adaptation process that can deal with the plant response to colonization in a more efficient manner.

Future perspectives

Frequent intimate interaction between human pathogens and plants may make many different plant species susceptible to adjusted strains and become secondary hosts. Eventually this could lead to a higher prevalence of *Salmonella* serovars inside produce so that these cannot be removed by sanitation procedures. 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. Nevertheless, the potential presence of human pathogens inside lettuce should be taken into

consideration as an emerging risk for human health.

From the tentative route of infection described in this thesis several critical points can be identified that can block or reduce the transmission to and colonization of produce by *Salmonella* serovars. The chemotaxis driven attraction might be disturbed by increasing microbial competition, for example by increasing the organic matter content of the soil. This could temporarily enhance available nutrients, potentially facilitating the survival of *Salmonella* serovars temporarily. However, in soil with active organic matter exudates would be masked and the protective layer of rhizosphere bacteria would be enhanced around the roots (Van Diepeningen *et al*., 2005; Van Diepeningen *et al*., 2006). Indeed, enteric pathogens were suppressed more in some organically than in conventionally managed soils (Franz *et al*., 2005). Moreover, preliminary results indicate that the presence of antagonists like *P. fluorescens* can reduce the number of *Salmonella*-colonized plants under axenic conditions (Franz, unpublished). Other results confirmed that the soil microflora is partially determining the endophytic microflora of a plant (Press and Phoenix, 2005; Yang and Crowly, 2000; Klerks, unpublished). Further improvement of the rhizosphere (and thus also the endosphere) by controlling the available root-colonizing bacteria that have antagonistic properties against human pathogenic bacteria, might therefore lead to a significant reduction of produce contamination. Another possibility for control of *Salmonella* lies in the genome of lettuce. In this thesis only three cultivars were evaluated for susceptibility for *Salmonella* serovars, already indicating differences with respect to susceptibility. Evaluation of a larger set of cultivars might present a clear differential resistance against *Salmonella* serovars (or human pathogenic bacteria in general) based on more effective defense systems. Plant breeding for resistance to human bacterial pathogens like *Salmonella* serovars in combination with improved rhizosphere communities would thus provide an easily accessible approach to farmers for prevention of contamination of produce.

The above mentioned strategies are hypothesized to reduce contamination during plant growth. However, preventive strategies at post-harvest or pre-cultivation might also lead to additive reductions in the number of contaminated plant- or produce-associated illness. Therefore, the reduction in produce-associated illness will gain profit from a whole chain approach. Such approach is difficult due to continuous changes in consumer food preference, food production and distribution practices (Sivapalaningam *et al.*, 2004). The globalization of the food supply also implies that spreading of contaminated produce is facilitated. Since agricultural practices and food processing differ between countries (think of sanitation procedures, use of sewer-contaminated irrigation water, etc.), this is believed to play an important role in produce-associated outbreaks. Even though, world-wide protocols for good

agricultural practices, food processing and robust monitoring-systems might be essential to reduce the number of outbreaks associated with produce. It is clearly evident that contamination of fresh produce with human pathogenic bacteria has become a serious threat for human health. Also, due to more intensive agriculture the likelihood of contamination of crops has increased (Franz *et al.*, 2005). To what extent this contributes to a higher incidence of *Salmonella* serovar-related disease outbreaks or even to more plant-adapted *Salmonella* serovars, is not yet known. Up to date preventive strategies to limit the occurrence of human pathogens in association with produce have not yet been applied extensively and internationally, even though factors like the feeding regime of cattle (Russell and Rychlik, 2001; Franz *et al.*, 2005) or the composting of manure (Pell, 1997) have been described to reduce or eliminate the number of pathogens. Robust and reliable detection methods applied in monitoring-systems will provide better insight in the occurrence, spread, accumulation and reduction of the pathogens throughout the whole production chain. In conclusion, taking into account the data obtained from this and previously published research, it can be stated that an international uniform legislation for, and monitoring of, the whole food production chain might be necessary to counter the emerging threat of human pathogens in fresh produce.

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Summary

Summary

Salmonella is among the most commonly known bacterial pathogens to cause human illness. Often Salmonellosis is associated with the consumption of contaminated foods like meat, eggs or egg products. However, during the last decades an increase of outbreaks is recognized to be caused by human pathogenic bacteria in association with fresh produce. The use of manure for production of vegetables, e.g. lettuce, contributes significantly to the risk of contamination of fresh produce. Enteric pathogens like *Salmonella* associated with manure can come in close contact with plants like lettuce, and a better understanding of the interaction between lettuce and *Salmonella* serovars during cultivation is necessary to be able to take preventive actions to reduce the risk for human health.

This thesis describes the development of detection methods of *Salmonella enterica* and *Escherichia coli* O157:H7 for routine diagnostic screening in the food production chain. Next to that, it describes the physiological and molecular interaction between *Salmonella* serovars and lettuce. Background information concerning *Salmonella* serovars in association with lettuce, like history of produce-associated outbreaks, approaches to detect the pathogen in food samples, pathogenesis, plant responses and the molecular interaction between plants and human pathogens, is discussed in Chapter one.

Chapter two concerns the comparison of different molecular methods to detect *S. enterica* (*invA*-gene) or *E.coli* O157:H7 (*stx*-1, *stx*-2 and *eae*-gene) with respect to sensitivity, precision and accuracy. Two basic methods were selected, both based on real-time Taqman PCR, a method that generates fluorescence upon specific DNA amplification. The increase in fluorescence during PCR is directly correlated to the amount of target DNA present after each amplification cycle. The detection and quantification methods were improved by the addition of a general internal amplification control (IAC), viz. comprised of DNA coding for green fluorescent protein (*gfp*), that allowed the identification of false negative results. The IAC provided insight in amplification efficiency and enabled a more accurate quantification. Implementation of the IAC did not affect the precision of the methods, although the sensitivity was reduced 10-fold. At least 1 pg of target DNA (equal to 200 CFU) was detected and quantified with high precision and accuracy. Qualitative detection was feasible even down to 10 fg of target DNA (equal to 2 CFU) per reaction using both methods in which the IAC was incorporated. The methods enable a reduction in assay time to two days to test food samples, compared to five days required for the standardized procedures.

To improve molecular methods to detect the pathogen in environmental substrates, five commercially available DNA extraction methods were evaluated in Chapter three with respect to DNA extraction efficiency of *S.* Enteriditis from soil, manure and compost. An internal procedural control (GIPC) for DNA extraction and amplification was developed. The GIPC was based on the same control DNA as used for the IAC in Chapter 1, incorporating *gfp* containing bacterial cells (*E. coli* harboring a gfp-plasmid) in the sample prior to DNA extraction. Inclusion of the GIPC permitted a more accurate quantification of *S.* Enteriditis after DNA extraction and amplification and reduced the possibility of false-negatives. Using this protocol, the optimal extraction method differed for soil (Mobio soil DNA extraction kit), manure (Bio101 soil DNA extraction kit) and compost (Mobio fecal DNA extraction kit). With each method, at least 2000 CFU of added S. Enteriditis/100 mg substrate could be detected by direct DNA extraction and subsequent *S. enterica* specific Taqman PCR. After bacterial enrichment, as little as 1 CFU/100 mg of original substrate was detected. Using this approach a more reliable quantification was obtained for *S. enterica* initially present in environmental substrates.

In Chapter four the physiological and molecular interactions between the human pathogenic *S.* Dublin and the commercially available mini Roman lettuce cv Tamburo are described. Investigation of the localization of *S.* Dublin on/in lettuce plants revealed the presence of significant populations on the surface and inside the plants. The latter was evidenced from significant residual concentrations after highly efficient surface disinfection (99.81%) and fluorescence microscopy of *S.* Dublin in cross-sections of lettuce at the root-shoot transition region. A reduction in biomass was observed upon colonization of lettuce plants with *S.* Dublin compared to water-inoculated plants. Next to this physiological response, there were clear differential gene expression profiles between non-colonized and colonized lettuce plants based on transcriptome analysis by cDNA-AFLP. To confirm the results, generally and differentially expressed genes were selected, identified by sequence analysis and analyzed by RT-PCR to present the specific gene expression profiles in time. Functional grouping of the expressed genes indicated a correlation between colonization of the plants and an increase in expressed pathogenicity-related genes. From these results it was evident that lettuce plants respond to the presence of *S.* Dublin at a physiological and molecular level. In addition, it was confirmed that *Salmonella* serovars can colonize the interior of lettuce plants, thus potentially imposing a human health risk when contaminated lettuce is processed and consumed.

The fact that the lettuce plants responded to the colonization by *Salmonella* serovars suggested that differences in susceptibility between cultivars or differences in colonization

efficiency between *Salmonella* serovars might be present. In Chapter five, the differential interaction of *S*. Typhimurium, *S.* Enteritidis, *S*. Dublin, *S.* Newport and *S.* Montevideo with lettuce cultivars Cancan, Nelly and Tamburo is presented, in terms of prevalence and degree of endophytic colonization of lettuce by the *Salmonella* serovars. Besides a significant interaction, significant differences among serovars, but not among lettuce cultivars, were obtained when lettuce was grown under axenic conditions. When grown on soil, all three evaluated serovars *S.* Typhimurium, *S.* Enteritidis and *S*. Dublin were able to colonize lettuce epiphytically, but to a lower extent than on axenically grown plants. Only *S.* Dublin was able to colonize the plants endophytically when these were grown on contaminated soil. Species richness and diversity of the endophytic microbial community, determined from DGGE gels with DNA from *Salmonella*-colonized lettuce Cancan and Nelly, were negatively correlated with the number of *Salmonella* CFU / gram of lettuce. No correlation was observed for cultivar Tamburo. Thus, the microflora of lettuce cultivars Cancan and Nelly appeared more antagonistic to *Salmonella* serovars than that of cultivar Tamburo.

Besides plant-associated colonization, also the active movement of *Salmonella* serovars towards lettuce roots was assessed. Movement was visualized using a metabolism marker (tetrazolium) for chemotaxis. Reduction of this marker suggested the presence of an organic compound in the lettuce root exudates that was used as carbon source by the *Salmonella* serovars. Subsequent micro-array analyses with DNA extracted from a broth culture of *Salmonella* with or without exudates identified genes of *S.* Typhimurium that were induced by root exudates. These genes, trehalose-6-phosphate synthase (*Ots*A; utilizes glucose-6-phosphate as substrate), hexose phosphate utilization protein (*Uhp*C; sensor for external glucose-6-phosphate), putative effector protein (*Ssa*H; regulator of secretion of the type III secretion system), and putative anti-silencer RNA (*Drs*A; regulator of transcription to express *rcs*A promoter, responsible for capsular polysaccharide synthesis), imply a relation with a sugar-like carbon source and thus suggest an association with chemotaxis. The results described in Chapter 5 reveal different plant and microbial factors that influence the colonization efficiency of *Salmonella* serovars. The serovar and cultivar, but indirectly also the rhizosphere and the endophytic microflora of lettuce were most influential with respect to the risk of colonization and thus the risk for human health.

Finally, an extensive discussion concerning the research of Chapters two to five is described in Chapter six, including future perspectives of risk for human health, route of infection and risk reduction in the production chain of *Salmonella*-associated lettuce.

Samenvatting

Samenvatting

Salmonella is één van de meest bekende ziekteverwekkende bacteriële pathogenen. Vaak is Salmonellosis geassocieerd met de consumptie van besmet voedsel zoals vlees, eieren of eiproducten. Echter, gedurende de laatste decennia heeft er een toename van ziekte-uitbraken plaatsgevonden geassocieerd met de consumptie van besmette verse groenten. Oorzaak van deze toename is meerledig, waarbij het gebruik van mest bij de productie van groenten zoals sla significant bijdraagt aan het risico van besmetting van verse groenten. De potentieel aanwezige pathogenen in mest, zoals *Salmonella*, kunnen hierdoor in contact komen met planten en groentengewassen. Om het risico voor de mensgezondheid te verlagen moeten preventieve maatregelen worden getroffen, waarbij inzicht in de interactie tussen *Salmonella* serovars en sla tijdens cultivatie essentieel is.

Dit onderzoek beschrijft de ontwikkeling van methoden voor detectie van *Salmonella enterica* en *Escherichia coli* O157:H7, toepasbaar in routinematige diagnostische evaluaties in de voedsel-productieketen. Daarnaast wordt de fysiologische en moleculaire interactie tussen *Salmonella* serovars en sla beschreven. Achtergrondinformatie met betrekking tot *Salmonella* serovars in associatie met sla, zoals geschiedenis van groente-geassocieerde ziekte-uitbraken, detectie benaderingen voor aantonen van het pathogeen in voedingsproducten, pathogenese, reacties van de plant en de moleculaire interactie tussen planten en humaan pathogenen, wordt besproken in Hoofdstuk 1.

Hoofdstuk twee presenteert een vergelijking van moleculaire methoden voor de detectie van *S. enterica* (het *invA*-gen) of *E. coli* O157:H7 (het *stx-1*, *stx-2* en *eae*-gen) op basis van gevoeligheid, precisie en nauwkeurigheid. Hieruit zijn twee methoden geselecteerd, beide gebaseerd op real-time Taqman PCR waarbij fluorescentie wordt gegenereerd in het geval van specifieke amplificatie. De toename van fluorescentie tijdens PCR is direct gekoppeld aan de hoeveelheid specifiek DNA dat aanwezig is na elke amplificatie cyclus. De detectie en kwantificeringsmethoden zijn vervolgens geoptimaliseerd door toevoeging van een interne amplificatiecontrole (IAC; bestaat uit DNA dat codeert voor green fluorescent protein (gfp)), waarmee vals negatieve resultaten konden worden geïdentificeerd. Uit dit onderzoek bleek dat de IAC meer inzicht in de amplificatie-efficiëntie kon geven en een meer accurate kwantificering mogelijk maakte. De implementatie van de IAC had geen invloed op de precisie van de methoden, hoewel de gevoeligheid een factor 10 was verlaagd. Tenminste 1 pg DNA van het doelorganisme (gelijk aan 200 kolonievormende eenheden; CFU) kon worden gedetecteerd en gekwantificeerd met een hoge nauwkeurigheid. Met beide

methoden was een kwalitatieve detectie van het doelorganisme zelfs mogelijk tot 10 fg DNA (gelijk aan 2 CFU) per PCR reactie wanneer ook de IAC was toegevoegd. De methoden maakte het mogelijk om de analysetijd voor het testen van voedingsproducten te reduceren tot twee dagen ten opzichte van vijf dagen die nodig zijn voor de gestandaardiseerde procedures.

Ter verbetering van moleculaire methoden om pathogenen te detecteren in substraten zoals grond, mest en compost, is in Hoofdstuk drie een vergelijking gemaakt tussen vijf DNA extractiemethoden met betrekking tot de DNA extractie-efficiëntie van *S.* Enteritidis uit deze substraten. Een generieke, interne procedure controle (GIPC) voor DNA extractie en amplificatie is ontwikkeld. De GIPC is gebaseerd op hetzelfde controle DNA als de IAC beschreven in Hoofdstuk 1. Echter in deze experimenten bestond de GIPC uit *E. coli* cellen met gfp-coderende plasmiden, welke aan het monster voor de DNA extractie werden toegevoegd. Additie van de GIPC maakte een nauwkeuriger kwantificering van *S.* Enteritidis mogelijk na DNA extractie en amplificatie en reduceerde de aanwezigheid van vals negatieve resultaten. Gebruikmakend van dit protocol bleken de meest optimale DNA extractiemethoden verschillend voor DNA extractie uit grond (Mobio soil DNA extraction kit), uit mest (Bio101 soil DNA extraction kit en uit compost (Mobio fecal DNA extraction kit). Met elk van deze methoden was het mogelijk minimaal 2000 CFU van toegevoegde *S.* Enteritidis / 100 mg substraat te detecteren na DNA extractie en *S. enterica* specifieke Taqman PCR. Na bacteriële verrijking was het zelfs mogelijk 1 CFU / 100 mg substraat te detecteren na DNA extractie en Taqman PCR. Door gebruik te maken van deze nieuwe benadering is een meer betrouwbare kwantificering van *S. enterica* in substraten als grond, mest en compost mogelijk geworden.

In Hoofdstuk vier worden de fysiologische en moleculaire interacties tussen humaan pathogene *S.* Dublin en de commercieel verkrijgbare Mini Romein sla cultivar Tamburo beschreven. Onderzoek naar de localisatie van *S.* Dublin op en in de slaplanten toonde de aanwezigheid van significante populaties *S.* Dublin aan. De aanwezigheid van *S.* Dublin in de plant werd bevestigd door significante hoeveelheden na efficiënte oppervlakte desinfectie (99,81%) en fluorescentie-microscopie van *S.* Dublin in doorsneden van de wortel-stengel overgang van sla. Een verlaging van de biomassa werd gevonden bij planten gekoloniseerd door *S.* Dublin, indien vergeleken met water-geïnoculeerde planten. Naast fysiologische reacties werden op basis van transcriptoom analyses met behulp van cDNA-AFLP ook differentiële gen-expressieprofielen gevonden tussen gekoloniseerde planten en niet-gekoloniseerde planten. Om deze resultaten te bevestigen zijn generiek en specifiek tot expressie gekomen genen geselecteerd, geïdentificeerd door middel van sequentie-analyse

en getest met RT-PCR om de specifieke gen-expressieprofielen in de tijd zichtbaar te maken. Functionele categorisatie van de tot expressie gekomen genen gaf een correlatie tussen de kolonisatie van sla en een toename van expressie van genen betrokken bij pathogeniteit. Op basis van deze resultaten was het evident dat slaplanten zowel op fysiologisch als op moleculair niveau reageren op de aanwezigheid van *S.* Dublin. Daarnaast is bewezen dat *S.* Dublin de slaplant via de binnenkant kan koloniseren, waardoor er in potentie een risico voor mensgezondheid bestaat wanneer gecontamineerde sla wordt verwerkt en uiteindelijk geconsumeerd.

Het feit dat slaplanten reageerden op kolonisatie met *Salmonella* serovars suggereert de aanwezigheid van verschillen in vatbaarheid tussen sla cultivars of verschillen in kolonisatie-efficiëntie tussen *Salmonella* serovars. In Hoofdstuk vijf wordt de differentiële interactie van *S.* Typhimurium, *S.* Enteritidis, *S.* Dublin, *S.* Newport en *S.* Montevideo met sla cultivars Cancan, Nelly en Tamburo gepresenteerd, in het bijzonder gericht op prevalentie en mate van endofytische kolonisatie van sla door de *Salmonella* serovars. Naast een significante interactie zijn tevens significante verschillen tussen de serovars, maar niet tussen de cultivars, aangetoond in het geval dat de slaplanten waren gegroeid onder axenische condities. In het geval dat de planten werden gegroeid op grond, koloniseerden de drie geteste serovars *S.* Typhimurium, *S.* Enteritidis en *S.* Dublin de planten epifytisch. Alleen *S.* Dublin kon de slaplanten ook endofytisch koloniseren indien de planten groeiden op gecontamineerde grond. De species rijkdom en diversiteit van de endofytische microbiële populatie, bepaald met behulp van DGGE gelen met DNA van *Salmonella* gekoloniseerde sla cultivars Cancan en Nelly, bleken negatief gecorreleerd aan het aantal geïsoleerde *Salmonella* cellen / gram sla. Voor cultivar Tamburo werd er geen correlatie gevonden. Op basis van deze resultaten lijkt het dat de microflora van sla cultivars Cancan en Nelly meer antagonistisch waren voor *Salmonella* serovars dan die van cultivar Tamburo.

Naast de plant-geassocieerde kolonisatie is ook de actieve verplaatsing van *Salmonella* serovars naar slawortels onderzocht. De verplaatsing werd gevisualiseerd door gebruik te maken van een metabolisme marker (tetrazolium) voor chemotaxis. Omzetting van deze marker suggereert de aanwezigheid van een organische stof in de wortelexudaten die als koolstofbron dient voor de *Salmonella* serovars. Met behulp van micro-array analyses van DNA geëxtraheerd uit een *Salmonella* cultuur met of zonder de wortelexudaten zijn genen van *S.* Typhimurium geïdentificeerd die differentieel tot expressie zijn gekomen door aanwezigheid van de exudaten. Deze genen, trehalose-6-phosphate synthase (*Ots*A; gebruikt glucose-6-phosphate als substraat), hexose phosphate utilization protein (*Uhp*C; sensor voor externe glucose-6-phosphate), putative effector protein (*Ssa*H; regulator van

secretie van het type III secretie systeem) en putative anti-silencer RNA (*Drs*A; regulator van transcriptie om de *rcs*A promoter tot expressie te brengen, die verantwoordelijk is voor capsulaire polysaccharide synthese), suggereren een relatie met een suikerachtige koolstofbron en impliceren dus een associatie met chemotaxis. De resultaten die beschreven zijn in Hoofdstuk vijf geven aan dat er verschillende plant- en bacteriefactoren een rol spelen en de kolonisatie-efficiëntie van *Salmonella* serovars beïnvloeden. Zowel de serovar als de cultivar, maar indirect ook de rhizosfeer en de endofytische microflora van de slaplant, droegen het meest bij aan het risico voor kolonisatie en dus het risico voor de mensgezondheid.

Als laatste is in Hoofdstuk zes een uitgebreide discussie toegevoegd betreffende het onderzoek beschreven in Hoofstukken twee tot en met vijf, inclusief een toekomstvisie van risico's voor mensgezondheid, de route van infectie en potentiële strategieën voor verlaging van het voorkomen van *Salmonella*-geassocieerde sla in de productieketen.

Nawoord

Nawoord

Na afronding van het HLO was het meteen duidelijk dat ik ooit wilde promoveren. Ik dacht dat werkervaring net zoveel zou tellen als een universitaire graad behalen. Maar hoe meer (werk)ervaring ik kreeg, des te duidelijker werd het dat de gekozen weg niet de meest gemakkelijke zou zijn. Het zou erop neerkomen dat ik zelf een project moest regelen om bij PRI te kunnen promoveren. Laten we het een samenloop van omstandigheden en geluk noemen dat ik met mijn idee bij Jan van der Wolf heb aangeklopt, die me vervolgens verwees naar Jim van Vuurde. Samen met Jim heb ik een voorstel geschreven, dat pas in het jaar daarna zou worden geaccepteerd. Toch hielden we vol en kwamen in de tussentijd in contact met Ariena van Bruggen, professor Biologische Bedrijfssystemen van Wageningen Universiteit. Het enthousiasme spatte er tijdens deze gesprekken van af en het project werd groter en groter. Helaas kon ik het traject niet samen met Jim afronden, maar gelukkig kon ik terugvallen op Jan van der Wolf. Dit was echter maar voor twee maanden. Jan stapte over naar een andere groep. Ik trok aan de noodklok, genaamd Carolien Zijlstra. Zij pakte het project goed op en heeft het samen met Ariena tot een gecombineerde STW en Produktschap Tuinbouw financiering gebracht. In totaal drie AIO's inclusief assistentie konden hiermee worden aangesteld. Wat een geluk en luxe om te kunnen promoveren op mijn eigen gekozen onderwerp. En wat een resultaat, om trots op te zijn! Wie zou ooit hebben gedacht dat *Salmonella* bacteriën in een plant zouden kunnen groeien en een respons zouden kunnen induceren? Op het moment dat het project startte, was het groepje 'believers' in ieder geval maar erg klein. Nu, na vier jaar, is deze groep aanzienlijk gegroeid en wordt het onderzoek naar humaan pathogenen in relatie met groenten nauwgezet gevolgd. Ik kan je zeggen, dat geeft een goed gevoel!

Net zoals bij elk promotieonderzoek is ook deze niet solistisch uitgevoerd en werd elke hulp zeer gewaardeerd. Op de allereerste plaats wil ik graag mijn promotor, professor Ariena van Bruggen, heel erg bedanken voor haar tomeloze enthousiasme en prikkelende hypothesen. De tijd die je genomen hebt voor het reviewen en geven van suggesties voor mijn manuscripten en deze thesis heb ik enorm gewaardeerd. De onderzoekssturende statistiek die je verwachtte van ons promovendi werd opeens een handige tool die experimenten en manuscripten veel waardevoller maakte. Ook de gezellige etentjes bij jou thuis samen met de collega's van je groep waren erg geslaagd. Hoewel ik bij PRI mijn werk uitvoerde, voelde ik me mede hierdoor toch een deel van jouw groep. Erg bedankt hiervoor! Ook wil ik Carolien Zijlstra als copromotor bedanken voor de vrijheid die ze me gelaten heeft tijdens mijn promotie, alsook voor het screenen van de manuscripten en deze thesis op taalfouten en 'ambiguities'. Thanks, Carolien!

Eelco, jij bent bijna tegelijk met mij gestart en jij zal ook binnenkort jouw proefschrift mogen verdedigen. Voornamelijk met de statistische vraagstukken kon ik goed bij je terecht, maar we ondersteunden elkaar ook bij onze experimenten. Zo hebben we een keer bij een boer aan de keukentafel ons onderzoek toegelicht, om vervolgens met de zakken vers geschepte mest achterin de auto weer door te rijden naar de volgende veehouder. Hoe dicht kun je bij de praktijk komen? Tijdens het ISME symposium in Oostenrijk was het erg gezellig en hebben we ons onderzoek goed kunnen presenteren. Je vrouw Joke heb ik daar ook leren kennen, nog voordat ze zwanger werd van jullie dochter Eline. Bedankt voor de goede, gezellige en gezamenlijke promotietijd en veel geluk toegewenst met je dames, Eelco!
Sasha Semenov, you had the sudden opportunity to join this project. Although we did not do many experiments together, I enjoyed your discussions and strong points during the microbiological meetings. Who would think of linking economical statistics to bacterial fluctuations? Thank you and good luck with your final year of this PhD. I would also like to thank Maxim Donnikov. Maxim, you guided me for two weeks through Moscow, which was cultural and above all very joyful. During your stay in the Netherlands you helped me extensively with all the DNA extractions, for which I thank you a lot. I will also never forget the visit to the famous Sint-Jan Cathedral in my birthplace ’s-Hertogenbosch. An orthodox Christian in a catholic cathedral; it must have felt like tress-passing. Unexpected, the Sint-Jan Cathedral had a special connection to your cathedral in Moscow. Coincidence, maybe? Also the Russian colleagues, Vladimir Mikhailovic, Alexander Semenov and Vladimir Zelenev I would like to thank for their kindness and unlimited joy and enthusiasm in research. I must say, my two-week visit to Russia was very impressive, Ariena van Bruggen(ova) knows where to find quality research! Uiteraard wil ik Aad Termorshuizen ook hartelijk danken. Rustig de zaken overdenkend en indirect richting gevend nam je deel aan de twee wekelijkse groepsdiscussies. Dit was erg plezierig en ik denk dat velen het jammer zullen vinden dat je recentelijk de universiteit hebt verruild voor het bedrijfsleven.
De meeste tijd heb ik echter mijn collega’s bij PRI onrustig gemaakt door met *Salmonella* te werken. Ik besef dat ik hiermee bij sommigen wat stress veroorzaakt kan hebben. Als er ‘*Salmonella*-project’ op het potje geschreven stond, bleef iedereen er in ieder geval ver bij uit de buurt. Sommigen bleven zelfs uit het lab als ik er was. Gelukkig waren er ook collega’s die ik over de psychologische drempel heb kunnen duwen, zoals Marga van Gent-Pelzer. Marga, je hebt me geweldig geholpen gedurende deze vier enerverende jaren. Je stond altijd klaar om mij te helpen, ook al waren het soms wel heel erg veel plantjes…. Bedankt Marga! Naast de ondersteuning bij praktisch werk is het altijd erg plezierig als je eens kunt ‘sparren’ met een naaste collega die vanuit een andere hoek tegen het project aankijkt. Hiervoor wil ik graag iedereen van de cluster Moleculaire Fytopathologie bedanken die heeft bijgedragen aan de sfeer en de uitdagende discussies tijdens vergaderingen of in de wandelgangen.

Theo van der Lee wil ik hierbij graag noemen. Ik heb de gesprekken met jou enorm gewaardeerd. Niet alleen vanwege je frisse en wetenschappelijke kijk op zaken, maar je stond ook altijd klaar als ik je mening nodig had. Arjen Speksnijder, ook jij hebt met jouw specialistische invalshoek vanuit de microbiële ecologie regelmatig met mij van gedachten gewisseld. Erg bedankt hiervoor! Cees Waalwijk, jou wil ik bedanken voor de goede samenwerking in projecten. Voordat je het weet zit je samen met een paar Finnen in een zweethokje vlak bij de poolcirkel. We hebben aan den lijve mogen ondervinden dat 'business in relaxation' inderdaad in een sauna plaatsvindt. Natuurlijk wil ik ook mijn kamergenoten Richard van Hoof en Margarit de Kleijn noemen. Op de spaarzame momenten dat ik mijn gedachten van het scherm kon trekken, hebben we het over veel dingen gehad. Hierbij voerden de little ones (Bram, Koos, Loek) de boventoon. Bedankt roomies! En Richard, jouw fotografische expertise is meer dan eens van pas gekomen voor mijn onderzoek, heel erg bedankt hiervoor! Ook mijn bovenburen wil ik niet ongenoemd laten. Jules Beekwilder, Francel Verstappen, Ric de Vos en Harro Bouwmeester, bedankt voor jullie enthousiasme en interesse in mijn ideëen en dat ik gebruik heb kunnen maken van jullie expertise voor analytische en biochemiosche analyses. Buiten PRI ben ik ook de collega's van het RIKILT, in het bijzonder Henk Aarts, Armand Hermans en Angela van Hoek, veel dank verschuldigd voor alle *Salmonella* stammen die op aanvraag uit de kast werden getrokken en opgekweekt. En natuurlijk niet te vergeten de micro-array analyses die bij jullie zijn uitgevoerd, waarbij het uiterste is gevergd van deze technologie.
Familie en vrienden wil ik graag bedanken voor hun steun en het aanhoren van de tomeloze woordenvloed die er volgde op de vraag hoe het gaat met het onderzoek. Mijn promotieonderzoek was een geijkt onderwerp onder het genot van een goede wijn en heeft regelmatig geleid tot enthousiaste gesprekken. Misschien is niet altijd alles begrepen, maar de essentie is overgekomen; eet gewoon verse groenten! In het bijzonder wil graag mijn goede vriend Ton van de Wiel bedanken voor de uitstapjes, het golfen (eindelijk is er nu tijd om het GVB te halen!) en de goede gesprekken als vrienden onder elkaar. Bedankt allemaal voor jullie 'nuchtere' ideeën!

Ten slotte wil ik me graag nog richten tot Tessa en Bram wie deze jaren intensief met mij hebben beleefd. Tessa, in vier jaar kan er veel veranderen in een leven, zo ook die van ons. Wij zijn getrouwd, hebben een lieve zoon Bram en ons tweede kindje is in aantocht. Je hebt me altijd onvoorwaardelijk gesteund en gestimuleerd in deze vier jaren. Regelmatig ben ik avonden en weekenden aan het werk geweest of was ik op reis, waardoor dit promotieonderzoek veel van onze vrije tijd heeft gekost. Toch zorgde jij ervoor dat, tijdens momenten dat het leven meer geleid werd door mijn werk, alles op rolletjes bleef lopen. Voor jou is dit niet altijd even gemakkelijk geweest, zeker niet toen je me tijdens de

zwangerschappen hard nodig had. Het heeft soms veel van ons gezin gevraagd, in ieder geval zoveel dat jij deze promotie net zo goed hebt verdiend. Lieve Tess, jij bent de vrouw van mijn leven! Lieve, stoere Bram, na een dag werken is het altijd geweldig om thuis te komen en jou naar me toe te zien rennen van blijdschap. Je bent voor mij een onuitputtelijke bron van energie die me elke dag weer opnieuw vult tot de rand. Jongen, papa is trots op je! Dan te bedenken dat in oktober ons tweede wonder geboren wordt. Ik kan niet wachten tot ik je broertje of zusje ook in mijn armen kan nemen! Een promotie met zo'n achterban maakt het verschil!

Michel

Curriculum Vitae

Curriculum vitae

Michel Mathijs Klerks werd geboren te ‘s-Hertogenbosch op 14 januari 1974. In 1992 behaalde hij zijn HAVO diploma aan het toenmalige Rijksscholengemeenschap. Van 1992 tot 1994 heeft hij in Eindhoven aan de Hogere Laboratorium Opleiding zijn Propedeuse gehaald. Daarna heeft hij tot 1997 de studie Medische Biotechnologie voortgezet bij het HLO Faculteit natuur en Techniek te Utrecht. Zijn afstudeerstage bij het toenmalige Organon Teknika te Boxtel heeft geleid tot co-inventorschap van een patent met betrekking tot een innovatieve nucleïnezuur extractie methode. In 1997 trad hij in dienst als research analist bij Organon Teknika waar hij werkte aan de ontwikkeling van magnetische nucleïnezuur extractie en de real-time detectie van nucleic acid sequence-based amplification (NASBA) met behulp van molecular beacon technologie. In 1998 is hij overgestapt naar het toenmalige IPO-DLO om daar (multiplex) detectie toetsen te ontwikkelen voor aardbeivirussen en andere plant pathogenen. In 2003 is hij bij Plant Research International BV (gefuseerd IPO-DLO) gestart met zijn promotieonderzoek in samenwerking met de Wageningen universiteit, leerstoelgroep biologische bedrijfssystemen. Daar verrichtte hij onderzoek naar methodieken voor detectie en kwantificering van *Salmonella* spp. en *Escherichia coli* O157:H7 toepasbaar in de groenten productieketen, alsook de fysiologische en moleculaire interactie tussen sla en *Salmonella* spp.. De resultaten hiervan zijn terug te vinden in dit proefschrift. Tijdens dit onderzoek heeft hij gedurende twee weken het Engelhardt Institute te Moskou bezocht om te werken aan on-chip PCR amplificatie in 3D micro-gelpads. Daarnaast was hij nauw betrokken bij en verantwoordelijk voor aanpalende projecten en initiatieven op het gebied van voedselgezondheid en nanotechnologie. Na afronding van het promotie-onderzoek in 2007 is hij als onderzoeker bij Plant Research International BV gebleven waar hij als moleculair microbioloog onderzoek verricht aan voedselgezondheid en innovatieve nanotechnologie-gebaseerde detectie methoden.

List of Publications

Scientific publications

Klerks, M. M., A. H. C. Van Bruggen, E. Franz, and C. Zijlstra. 2007. Differential interaction of human pathogenic *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. *Submitted for publication*.

Franz, E., M. M. Klerks, O. J. De Vos, A. D. Van Diepeningen, A. J. Termorshuizen, and A. H. C. Van Bruggen. 2007. Farm prevalence of STEC *stx1*, *stx2*, *eaeA* and *rfbE* genes and survival of *E. coli* O157:H7 in manure from organic and low-input conventional dairy farms. Appl. Env. Microbiol. **73:**2180-2190

Klerks, M. M., M. Van Gent-Pelzer, E. Franz, C. Zijlstra, and A. H. C. Van Bruggen. 2007. Physiological and molecular response of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin. *Accepted by* Appl. Env. Microbiol.

Franz, E., A. A. Visser, A. D. Van Diepeningen, M. M. Klerks, A. J. Termorshuizen, and A. H. C. Van Bruggen. 2006. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Food Microbiol. **24:**106-112

Klerks, M. M., A. H. C. Van Bruggen, C. Zijlstra, and M. Donnikov. 2006. Comparison of methods to extract *Salmonella enterica* DNA from environmental substrates and quantification using a general internal procedural control. Appl. Env. Microbiol. **72:**3879-3886.

Schoen, C. D., M. Szemes, P. J. M. Bonants, A. Speksnijder, M. M. Klerks, P. H. J. F. Van Den Boogert, C. Waalwijk, J. M. Van Der Wolf, and C. Zijlstra. 2005. Novel molecular and biochemical techniques for quality control and monitoring in the agrofood production chain. *In:* Rapid Methods for biological and chemical contaminants in food and feed. A. van Amerongen, D. Barug & M. Lauwaars (Eds). Wageningen Academic Publishers, The Netherlands, pp 151-177.

Klerks, M. M., C. Zijlstra, and A. H. C. Van Bruggen. 2004. Comparison of real-time PCR methods for detection of *Salmonella enterica* and *Escherichia coli* O157:H7, and quantification using a general internal amplification control. J. Microbiol. Methods **59:**337-349.

Bonants, P. J. M., C. D. Schoen, M. Szemes, A. Speksnijder, M. M. Klerks, P. H. J. F. Van Den Boogert, C. Waalwijk, J. M. Van Der Wolf, and C. Zijlstra. 2004. From

single to multiplex detection of plant pathogens;pUMA, a new concept of multiplex detection using micro-arrays. Phytopathol. Pol. **35:**29-47.

Vaskova, D., J. Spak, M. M. Klerks, and C. D. Schoen. 2004. Real-time NASBA for sensitive detection of *Strawberry vein banding virus* and its comparison to PCR. Eu. J. Plant Pathol. **110:**213-221.

Klerks, M. M., J. L. Lindner, D. Vaskova, J. Spak, J. R. Thompson, W. Jelkmann, and C. D. Schoen. 2004. Detection and tentative grouping of Strawberry crinkle virus isolates. Eu. J. Plant Pathol. **110:**45-52.

Thompson, J. R., S. Wetzel, M. M. Klerks, C. D. Schoen, D. Vaskova, J. Spak, and W. Jelkmann. 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in *Fragaria vesca* L. J. Virol. Methods **111:**85-93.

Van Vuurde, J. W. L., M. M. Klerks, J. Kohl, C. Zijlstra, J. D. Van Elsas, and L. S. Van Overbeek (2002). Incidence and relevance of human pathogenic bacteria and fungi in fresh market vegetables. Internal review Plant Research International.

Klerks, M. M., and G. Leone. 2002. Use of AmpliDet RNA to control the microbiological quality of agro-food products. Agro Food Industry hi-tech **5:**37-40.

Goncalves, M. C., M. M. Klerks, M. Verbeek, J. Vega, and J. F. J. M. Van Den Heuvel. 2002. The use of molecular beacons combined with NASBA for the sensitive detection of *Sugarcane yellow leaf virus*. Eu. J. Plant Path. **108:**401-407.

Szemes, M., M. M. Klerks, J. F. J. M. Van Den Heuvel, and C.D. Schoen. 2002. Development of an AmpliDet RNA assay for the specific detection of PVY^{NTN}. J. Virol. Methods **100:**83-96.

Klerks, M. M., G. O. M. Leone, J. L. Lindner, C. D. Schoen, and J. F. J. M. van den Heuvel. 2001. Rapid and sensitive detection of *Apple stem pitting virus* in apple trees through RNA amplification and probing with fluorescent molecular beacons. Phytopathology **91:** 1085-1091.

Klerks, M. M., J. F. J. M. Van Den Heuvel, and C. D. Schoen. 2001. Detection of nematode-transmitted nepoviruses by the novel, one-tube AmpliDet RNA assay. Acta Hort. **550:**53-58.

Klerks, M. M., G. O. M. Leone, M. Verbeek, J. F. J. M. van den Heuvel, and C. D. Schoen. 2001. Development of a multiplex AmpliDet RNA for the simultaneous detection of *Potato leafroll virus* and *Potato virus Y* in potato tubers. J. Virol. Methods **93:**115-125.

Klerks, M. M., C. D. Schoen, and C. Waalwijk. 2000. The detection of trichodiene synthase mRNA in Fusarium on seeds using AmpliDet RNA. MITTEILUNGEN-BIOLOGISCHEN BUNDESANSTALT FUR LAND UND FORTWIRTSCHAFT **377:**49

Abstracts and publications aimed at scientific public

Franz, E., A. J. Termorshuizen, M. M. Klerks, A. D. Van Diepeningen, A. V. Semenov, O. J. De Vos, and A. H. C. Van Bruggen. 2006. Ecology of E. coli O157:H7 in the primary production chain of lettuce and modeling risks. *In: Proceedings of the 6th International symposium on Verotoxin producing Escherichia coli infections, Melbourne, Australia, 2006. Melbourne, Australia: p. 57.*

Franz, E., M. M. Klerks, A. J. Termorshuizen, and J. Bokhorst. 2006. Pathogenen in de primaire productieketen van bladgroenten. *Infectieziekten bulletin 17 (11): p. 381 - 382.*

Klerks, M. M., M. Van Gent-Pelzer, E. Franz, A. Van Bruggen, and C. Zijlstra. 2006. Colonization and interaction of lettuce with human pathogenic Salmonella *spp.*. *In: Proceedings of the International symposium of Salmonella and Salmonellosis, Saint-Malo, France. Saint-Malo, France: p. 417-418.*

Klerks, M. M., M. Van Gent-Pelzer, C. Zijlstra, A. J. Termorshuizen, and A. H. C. Van Bruggen. 2006. Physiological and molecular interactions between Salmonella *spp.* and lettuce. *In: 11th International Symposium on Microbial Ecology – ISME-11, Vienna, Austria. Vienna, Austria: p. 344.*

Klerks, M. M., M. Van Gent-Pelzer, C. Zijlstra and A. H. C. Van Bruggen. 2005. Analysis of interaction between Salmonella *spp.* and lettuce cultivars. In: *12th International Congress on Molecular Plant-Microbe Interactions, Mérida, Mexico. Mérida, Mexico: p. 196-197.*

Bonants, P. J. M., C. D. Schoen, M. Szemes, A. G. C. L. Speksnijder, M. M. Klerks, P. H. J. F. Van Den Boogert, C. Waalwijk, J. M. Van Der Wolf, and C. Zijlstra. 2004. From single to multiplex detection of plant pathogens by micro-arrays. *In: 4° Congresso da Sociedade Portuguesa de Fitopatologia, Universidade do Algarve, Faro, Portuguesa, 4-6 de Fevereiro de 2004. Sociedade Portuguesa de Fitopatologia: p. 72.Portuguesa: Universidade do Algarve: p. 72.*

Patents:

Gemen, B., Van Schijndel, H. B., and M. M. Klerks. 1999. Improved method for the isolation of nucleic acid. *Publication nr.:* WO/1999/039000.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (5 credits)

- Incidence and relevance of human pathogenic bacteria and fungi in fresh market vegetables (2003)

Writing of Project Proposal (7 credits)

- Detection of human pathogenic micro-organisms and their molecular interaction with freshly consumed vegetables (2003)

Laboratory Training and Working Visits (3 credits)

- Use of micro-arrays for detection of pathogens in soil; Engelhardt Institute (2003)
- Use of electric chip device for detection of *Salmonella* spp.; Institut fur siliconen technologi (ISIT) (2006)

Post-Graduate Courses (3 credits)

- Scientific writing level 5; WUR/CENTA (2003)
- Basic statistics; WUR/PE&RC (2003)

Deficiency, Refresh, Brush-up and General Courses (2 credits)

- Electron microscopy training; Virology/WUR (2001)
- Radiation hygiene level 5B; University Utrecht (1997)

Competence Strengthening / Skills Courses (3 credits)

- Acquisition training; WUR (2002)
- 360 degree feedback and assessment center; HFM (2006)

Discussion Groups / Local Seminars and other Scientific Meetings (8 credits)

- Biological farming system: Agricultural production systems (2003-2004)
- Bacteriology meetings (2005-2007)
- PRI: Molecular phytopathology meetings (2003-2007)
- PRI: Business unit meetings (2003-2007)
- EU-project progress meetings (2003-2007)

PE&RC Annual Meetings, Seminars and PE&RC Weekend (2 credits)

- Willie Commelin Scholten symposium (2003)
- WUR workshop human pathogens in the vegetable food chain (2006)

International Symposia, Workshops and Conferences (8 credits)

- Dutch society of microbiology (NVvM) (2005)
- IOBC meeting multitrophic interactions in soil and integrated control (2005)
- 12th Molecular plant Microbe interactions congress (2005)
- International symposium Salmonella and Salmonellosis (2006)

10th International symposium microbiological ecology (2006)