Ecology and Risk Assessment of *E. coli* O157:H7 and *Salmonella* Typhimurium in the Primary Production Chain of Lettuce

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Chapter 1

General introduction

1.1 Background

Food safety has become a major issue in the agri-food chain in the last decades. Foods can be contaminated by physical, chemical and biological hazards. With respect to risk perception, biological hazards seem to have a more notorious impact on public opinion than physical or chemical hazards (105). This is probably because biological risks are more frequently reported, can affect a large number of consumers and generally induce acute symptoms. A recent literature survey of human pathogens listed more than 1400 potential food-contaminating species, of which 58% is known to be zoonotic (i.e. pathogens which can naturally be transmitted between vertebrate animals and humans) (142). Among these 1400 pathogens, 538 (38%) are bacterial species, including 54 (10%) classified as emerging or re-emerging pathogens. An emerging pathogen can be defined as a pathogen whose incidence is increasing following its first introduction into a new host population; a re-emerging pathogen is one whose incidence is increasing in an existing host population due to changes in its epidemiology or environmental conditions (141). Interestingly, among the 177 pathogens classified as (re)emerging, 130 (74%) are zoonotic, which suggests that zoonotic pathogens are disproportionately likely to be associated with emerging and re-emerging infectious diseases. This association is the strongest for bacteria. These zoonotic (re)emerging pathogens are generally characterized by broad host ranges and these pathogens seem to exploit almost any change in human ecology that provides new opportunities for transmission (142). Most zoonotic pathogens are only moderately transmissible between humans but the magnitude of an outbreak can be very sensitive to changes in the basic reproduction number (i.e. transmissibility between humans) as affected by small alterations in the nature of the host-pathogen interaction (142) (Fig. 1). During 2005, 5311 foodborne disease outbreaks were reported by 23 European Union member states (1.18 outbreaks per 100000 inhabitants) (7). In the Netherlands, 44 outbreaks occurred that year (0.27/100000), which affected 321 people).

Escherichia coli O157:H7 and *Salmonella* are considered to be emerging zoonotic pathogens (121). The major reservoir of these pathogens consists of (healthy) agricultural animals, from which they spread to an increasingly variety of food. Traditionally, these pathogens are mostly associated with food products of animal origin like meat and eggs. However, recent outbreaks of foodborne disease associated with the consumption of fresh produce, like vegetables and fruits, raised concern that these products may be an increasing source of foodborne infections (114, 120). Currently, no risk assessment studies on the potential contamination of freshly consumed vegetables with human pathogenic bacteria are available. Detailed knowledge on the ecology of these pathogens in the primary production chain is needed in order to identify risk factors for their spread and survival. Subsequently, this knowledge can be used for the development of intervention strategies. The primary goal of this thesis is to contribute

to these knowledge gaps. This thesis is focused on *E. coli* O157:H7 and *Salmonella enterica* because of their status as emerging pathogens and their strong association with produce-related outbreaks (114). Lettuce (*Lactuca sativa*) was selected as model crop because of its importance as vehicle for the pathogens of concern (114) and because it is consumed raw. The latter imposes a higher potential health risk compared to crops which are cooked before consumption.

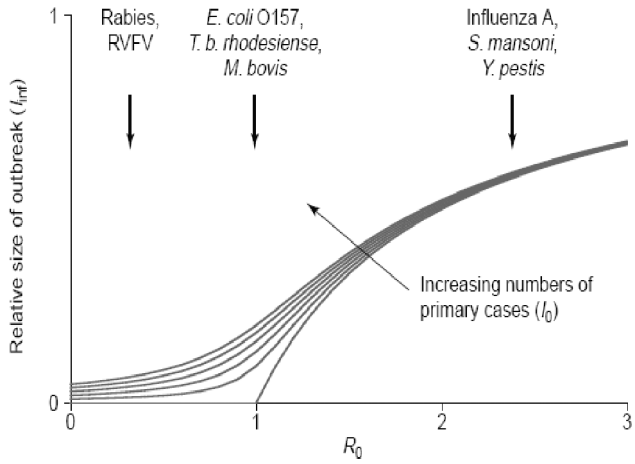

Fig. 1. Expected relationship between outbreak size (as fraction of the population affected) and the number of primary cases of infection introduced into the human population from an external source (zoonotic reservoir) (I0) and the basic reproduction number (R0), which is a measure of transmissibility of the infection between humans. Adapted from (141).

1.2 *Salmonella* spp.

Salmonella species are probably the most well-known bacterial foodborne pathogens. They are Gram negative, facultative anaerobic, rod-shaped, non-sporeforming, motile bacteria which belong to the family of Enterobacteriaceae. They can metabolize a wide variety of organic substrates by both respiratory and fermentative pathways. The genus *Salmonella* encompasses a large taxonomic group with over 2463 recognized serovars (63), which are classified according to biochemical characteristics and the immunoreactivity of two surface structures, the O and H antigens. The O antigen represents a *Salmonella* specific polysaccharide and the H antigen represents the filamentous portion of the bacterial flagella. Variation in these structures results in different classification within the *Salmonella* genus. The *Salmonella* genus consists of 2 species: *S. bongori* and *S. enterica*. The latter includes 6 subspecies: *S. enterica* ssp. *houtenae*, *arizonae, diarizonae, enterica,*

salamae and indica. *S. enterica* spp. *enterica* includes the human pathogenic *Salmonella* and consists of more than 2000 serovars including Typhimurium (of which more than 500 phage types are recognized), Typhi, Dublin, Enteritides, Montevideo, Newport etc.). The common nomenclature used in the scientific literature is the genus name directly followed by the serovar, like *Salmonella* Typhimurium instead of *Salmonella enterica* subspecies *enterica* serovar Typhimurium. *Salmonella* spp. are resilient bacteria and can adapt to extreme environmental conditions: (54).

1.2.1 Epidemiology

The disease caused by *Salmonella* is called salmonellosis and the two main manifestations of the disease are typhoid or typhoid-like fever and gastroenteritis. Of all 5311 foodborne disease outbreaks reported among 23 European Union Member States (MS) in 2005, 64% was due to *Salmonella* spp. (7). The most common serovars involved were Enteritidis (64%) and Typhimurium (2.5%), all other serovars showed an incidence of less than 1%. In the Netherlands, an estimated 50,000 people suffered from Salmonellosis between 1999 and 2000 and in 2002 this was approximately 35,000 (29). Total costs of salmonellosis in the Netherlands is estimated between 33 and 91 million euros annually (134). In the United Sates *Salmonella* causes approximately 1,400,000 cases annually, including 16,000 hospitalizations and 550 fatalities, which is 31% of the total foodborne disease cases (86).

The infectious dose of *Salmonella* spp. was thought to be in excess of 10,000 cells but a number of outbreaks have been reported where the infectious dose was found to be very low (10-100 cells), depending on the type of food, strain type, the physiological state of bacteria and characteristics of the host (41). The establishment of a human *Salmonella* infection depends on the ability to survive the environment outside the digestive system, the ability to survive the gastric acid of the human stomach and the ability of the pathogen to attach (colonize) and enter (invade) intestinal cells. For the latter, *Salmonella* must compete with indigenous gut microorganisms for suitable attachment sites. Diarrhoea associated with salmonellosis is thought to appear in response to bacterial invasion of intestinal cells rather than the action of enterotoxins. A main difference with other bacterial intestinal pathogens like *Shigella* and *E. coli,* who are replicating within the cytoplasma of host cells, is that *Salmonella* is confined to endocytotic vacuoles in which bacterial replication takes place. The infected vacuoles move and release *Salmonella* cells into the tissue. Prior to invasion of intestinal cells, *Salmonella* has to encounter and attach to these cells. This involves several types of fimbriae or pili (41). Genes coding for these fimbriae are located on the chromosome and on plasmids. Other virulence factors of *Salmonella* include siderophores (to retrieve essential iron from the host) and enterotoxins.

1.2.2 Reservoirs

Various agricultural animals can form a reservoir for *Salmonella*. These animals normally carry the pathogen asymptomatically. In the Netherlands, the most prominent isolated *Salmonella* serovars during the period 1984-2001 were Typhimurium and Enteritidis (respectively 44% and 24% of all *Salmonella* isolates) in humans, Dublin (53%) and Typhimurium (39%) in cattle, Typhimurium (69%) in pigs and Typhimurium (18%), Infantis (14%) and Enteritidis (12%) in chickens (129). Because of its surplus, cattle manure is the most used fertilizer in the Netherlands and therefore the focus of this thesis. The prevalence of *Salmonella* spp. in Dutch dairy herds in 1998, 1999, 2000, 2001 and 2002 was estimated at respectively 3%, 2%, 1%, 8.7% and 5.4% (29).

The most prominent serovar isolated in Dutch dairy herds was Typhimurium, which is recognized as a worldwide cattle and human pathogen with a principle reservoir in cattle (21, 34, 136). During the last decade, an increased prevalence of *Salmonella* Typhimurium phage type DT104 is recorded in several countries, including the UK (34) and the Netherlands (132). Approximately 30% of the human cases of salmonellosis in the Netherlands caused by *Salmonella* Typhimurium DT104 originated from cattle (133). Because of the strong association with human *Salmonella* infection and the cattle reservoir it was decided to focus the *Salmonella* research within this thesis on serovar Typhimurium.

1.3 *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram-negative, facultative anaerobic, common inhabitant of the gastrointestinal tract of mammals and belongs to the family of *Enterobacteriaceae*. Its niche is the mucous layer of the mammalian colon where it is thought to exploit its ability to utilize gluconate more efficiently than other resident species, thereby occupying a highly specialized niche (73). Most *E. coli* are harmless but a small proportion can cause clinical symptoms in humans and other mammals. Six main pathotypes of *E. coli* can be distinguished: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) and enterohaemorrhagic *E. coli* (EHEC). The various pathotypes of *E. coli* tend to be clonal groups that are characterized by shared O (lipopolysacharide) and H (flagellar) antigens that define serogroups (O antigen only) or serotypes (O and H antigens). All the pathogenic *E. coli* use a multi-step scheme of pathogenesis, which consists of colonization of the mucosal site, evasion of host defences and, multiplication and host damage (73). EHEC is the only pathogroup that has a definite zoonotic origin,

with cattle being recognized as the major reservoir (35). The best known and therefore often named the prototype EHEC strain is *E. coli* O157:H7, first identified in 1982 as a causative agent of bloody diarrhoea (haemorrhagic colitis, HC) and haemolytic uremic syndrome (HUS) in humans and associated with the consumption of undercooked beef (103).

1.3.1 STEC and EHEC

Early work in the 1970s demonstrated that certain *E. coli* strains could produce a toxin, which was initially called Verotoxin because of its distinct effect on Vero cells (kidney epithelial cells extracted from African green monkey) (74). These toxins were subsequently also called Shiga toxins (Stx) because of the close relation to the toxin produced by *Shigella dysenteria* type 1. The group of *E. coli* strains producing these toxins is referred to as Verocytotoxin producing *E. coli* (VTEC) or Shiga-toxin producing *E. coli* (STEC). These names are used interchangeably but the term STEC is used throughout this thesis. EHEC constitutes a subset of STEC that has appeared to be firmly associated with clinical symptoms and disease in human, like HC and HUS in humans (35). The mechanism of pathogenicity is not fully clear but is associated with the ability to produce toxins and the formation of attaching and effacing (AE) lesions (Paton & Paton 1998).

1.3.2 STEC virulence factors

The pathogenicity of STEC is determined by the presence of several virulence factors which are encoded by chromosomal pathogenicity islands, phage chromosomes integrated in the bacterial genome and plasmids.

One of the key virulence factors of STEC is the ability to produce Shiga-toxins (Stx) which consist of two types: Stx1 and Stx2. The toxins are produced by the pathogen in the colon and besides causing local damage, they can travel via the bloodstream to the kidney where it is thought to play a role in causing HC and HUS (73). The damage caused by the toxins is due to inhibition of protein synthesis which leads to necrosis and/or apoptosis of endothelial cells (98). The *stx1* and *stx2* genes are encoded on bacteriophage genomes integrated into the bacterial genome (prophages) and are under control of phage genes. *Stx*-phages are considered to be highly mobile genetic elements which can result in horizontal gene transfer of *stx* genes to *E. coli* and other Enterobacteriaceae (60). The expression of the *stx* genes (especially *stx2*) is affected by various environmental and stress conditions like temperature (Muhldorfer 1996, McIngvale 2002, Palumbo 1995), aeration (Leenanon 2003, McIngevale 2002), acid adaptation and starvation (Leenanon 2003, Naim 2006).

Table 1. Rapid Alert notifications concerning microbial contamination of fresh produce from 2004-2007. (http://ec.europa.eu/food/food/rapidalert/index_en.htm)

Date	Notified by	Reason	Origin
19-07-2004	UK	*Salmonella* in alfalfa, broccoli and radish sprouts	UK
26-11-2004	Sweden	*Salmonella* Thompson in rucola lettuce	Italy
30-11-2004	Sweden	*Salmonella* Thompson in rucola salad	Italy
03-12-2004	Denmark	*Salmonella* in rucola lettuce	Italy
08-12-2004	Slovenia	*Salmonella* Napoli in rucola lettuce	Italy
17-12-2004	Sweden	*Salmonella* typhimurium in ruccola lettuce	Italy
02-02-2005	Sweden	*Salmonella* in rucola lettuce	Italy
22-06-2005	Finland	*Salmonella* typhimurium DT 104 in iceberg salad	Spain
28-07-2005	Finland	*Salmonella* Aberdeen in fresh water spinach	Thailand
23-08-2005	Iceland	*Salmonella* Bareilly in baby asparagus	Thailand
07-09-2005	Finland	*Salmonella* Saint Paul and *Salmonella* Stanley in fresh water spinach	Thailand
14-10-2005	Finland	*Salmonella* Zanzibar in fresh lemongrass	Thailand
18-10-2005	Norway	*Salmonella* spp in fresh Chinese celery	Vietnam
18-10-2005	Norway	*Salmonella* spp in fresh celery and parsley	Vietnam
19-12-2005	Finland	*Salmonella* Zanzibar in fresh baby corn	Thailand
31-03-2006	Norway	*Escherichia coli* O157:H7 in paprika rosen	Spain
24-04-2006	UK	*Salmonella* spp in morning glory (water spinach)	Thailand
18-09-2006	Iceland	Suspected *Escherichia coli* O157:H7 in fresh baby spinach salad	United States
07-11-2006	Finland	*Salmonella* spp in rucola lettuce	Italy
09-11-2006	Finland	*Salmonella* group B in fresh water spinach	Thailand
14-11-2006	Norway	*Salmonella* Houtenae in rucola lettuce	Italy
24-11-2006	Finland	*Salmonella* Newport in fresh water spinach	Thailand
29-11-2006	Finland	*Salmonella* group C in fresh lemongrass	Thailand
10-01-2007	Finland	*Salmonella* Hvittingfoss in fresh water spinach	Thailand
14-03-2007	UK	*Salmonella* in canteloupe melon	Costa Rica
23-04-2007	UK	*Salmonella* (presence /25g) in lambs lettuce	UK

In addition to Shiga toxins, a whole cluster of virulence factors encoded by a chromosomal region called the locus of enterocyte effacement (LEE) is present in many STEC strains. These factors are responsible for the attaching and effacing lesions typical of many STEC strains. The LEE encodes for a type III secretion factor, an adhesion called intimin (*eae*) and for the translocated receptor of intimin (91). LEE-negative STEC are rarely associated with cases of severe human disease but some strains may possess other adherence mechanisms allowing them to colonize the intestinal mucosa as effective as the LEE-positive strains and consequently cause disease (for example STEC O111:H2 and O113:H21) (Caprioli 2005). The ability of STEC to produce AE lesions is probably sufficient to cause non-bloody diarrhoea but *stx* is essential for the development of bloody diarrhoea, HC and HUS (Nataro 1998). Like the genes encoding for the Shiga-toxins, the intimin gene and the entire LEE can be spread horizontally but this seems to be a rare event and the presence of LEE is strongly associated with particular STEC lineages (26).

Finally, a large plasmid (EHEC-hemolysin plasmid) encoding several putative virulence factors (like hemolysin) can also be found in a large proportion of STEC associated with disease (98).

It is generally considered that *E. coli* O157:H7 is more virulent than other STEC, although the reason for this is unclear (135). The high virulence of STEC strains like *E. coli* O157:H7 is not only determined by genes coding for toxins and adherence factors but partially also by its ability to survive environmental stress conditions. Their capacity to colonize the human gut is for a large part due to their resistance to low pH levels like encountered in the human stomach, which contributes to its very low infectious dose of approximately 50-100 cells or even lower (16). Environmental stress may modulate bacterial virulence by inducing cross-protection to other stresses resulting in increased survival (for example starvation stress can induce increased resistance to low pH levels, osmotic stress and antibiotics), adaptive mutations which will lead to increased survival and /or proliferation in stress-full environments, and enhanced expression of VHEC virulence factors like Shiga-toxins (37).

1.3.3 Evolution and emergence of STEC O157

Molecular analysis suggests that EHEC acquired the majority of its virulence genes by horizontal transfer of genetic material. The acquisition of the LEE pathogenicity island and *stx* genes were two crucial steps in the evolution of STEC O157 from a commensal ancestor (102). Phylogenetic analysis revealed that the lineages destined to give rise to *E. coli* K-12 and *E. coli* O157:H7 separated from a common ancestor as long as 4.5 million years ago and that old lineages of *E. coli* have gained the same virulence factors in parallel (102). The latter indicates that natural selection has

favoured an ordered acquisition of genes and the progressive build-up of molecular mechanisms that increase virulence, which probably enabled them to occupy new niches.

The emergence and increased prevalence of STEC O157 since its first description in 1982 is suggested to be associated with changing production and processing practices or changing consumer practices, or both. Understanding these reasons might help in the development of efficient control strategies. Higher cattle densities and increased cattle movements are thought to have contributed to the emergence and spread of this pathogen. Associated changes in cattle management practices might include the use of ionophores, a feed ingredient that is used to increase production efficiency by increasing nitrogen and carbon retention in the cattle digestive system (32). Ionophores, which are fermentation products of several actinomycetes, inhibit Gram-positive bacteria and may thereby promote Gram-negative bacteria like *E. coli* (69). It has also been suggested that STEC O157 may have been favoured in animals that were fed high carbohydrate diets (135). *E. coli* does not produce enzymes that can degrade plant polysaccharides (cellulose, hemiocellulose and starch), but can utilize soluble carbohydrates (like maltose) which are produced by starch-degrading bacteria (107). Cattle fed large amounts of raw corn had approximately 1000-fold more generic *E. coli* than cattle fed hay (44). In addition, these *E. coli* were much less sensitive to an acid-shock mimicking the human stomach, which is probably due to the fact that starch fermentation can cause a significant increase in volatile fatty acid concentrations and a decrease in pH (44). It is suggested that STEC O157 was subsequently able to spread relatively easily in large densely populated cattle systems, and is now found in all types of cattle production systems with no particular preferences (135). It has also been hypothesized that the increased use of manure to fertilize pastures may have played a role in the emergence of STEC since it increases the exposure of cattle to the pathogen (16). Finally, the decline in naturally occurring brucellosis (*Brucella* spp.) has been suggested to account for the emergence of STEC O157 since the O157 antigen cross reacts with antigens of *Brucella* (118). The lower host immunity towards *Brucella* might have promoted STEC O157.

Several studies showed a large diversity in the distribution of virulence factors among STEC strains (25, 27, 100). Most of the virulence properties of STEC are located on (semi)mobile elements like pathogenicity islands and plasmids which can be gained and lost relatively easily. As a result, the further emergence of new STEC or new variations by the horizontal spread of these genetic elements is likely to be a dynamic and ongoing process (135). Among over 100 STEC serotypes that have been isolated from humans, serotypes O157:H7 and O157:H$^-$ represent the majority of isolated strains associated with disease (27). It has been demonstrated with multivariate analysis that intimin (*eae*) and *stx2* are most strongly associated with disease in humans (27).

In contrast, *stx1* showed a significant association with bovine STEC serotypes. Since the distribution of virulence factors differs between human and bovine serotypes, these results suggest that STEC strains isolated from humans form a different population (or sub-population) than those isolated from the bovine reservoir (27). There is also evidence for the existence of two different *E. coli* O157 lineages, with one of the lineages being less virulent for humans or being less efficiently transmitted from the bovine reservoir to humans (46, 75).

1.3.4 Disease incidence due to STEC infection

STEC and *E. coli* O157:H7 in particular have been categorized as emerging foodborne pathogens (4, 121). STEC infections occur worldwide but are most common in the USA and Canada (91). *E. coli* O157:H7 alone is estimated to cause 73,480 illnesses in the United States annually, with approximately 2168 hospitalizations and 61 deaths (86). The associated economic costs were estimated to be 405 million US dollars (55).

In Europe, approximately 14,000 cases over 24 countries occurred between 2000 and 2005, of which 62% were serogroup O157 (50). The trend in STEC cases is increasing, with 2026 cases in 2000 compared to 2636 cases in 2005 (an increase of 30%). The O157 cases increased from 1443 to 1637 (+13%) while the non-O157 cases increased from 467 to 760 (+63%) (50). This probably merely reflects the adaptation of diagnostic tools that can detect non-O157 serotypes.

In the Netherlands 93 symptomatic cases of STEC O157 were reported between 1999 and 2001 (36 in 1999, 43 in 2000 and 14 in 2001) (130) and seem to be of limited public health interest. The annual incidence of STEC O157 infections in the Netherlands is estimated to be 0.26 per 100000 inhabitants, which is approximately 43 reported cases per (131). This level of incidence places the Netherlands, along with other European countries like Austria, Finland, Italy and Spain, among those with a relatively low occurrence of STEC O157 (130). Higher incidences are found in Germany, Sweden, England and Wales (1-2.5 per 100000) (5). Scotland has the highest incidence in Europe (4-10 per 100000) (68). Although the incidence of *E. coli* O157 in the Netherlands is low, STEC incidence should continuously be monitored because of the following reasons: 1) the very low infectious dose and potential severe clinical symptoms make STEC strains high-risk pathogens, 2) the significance of non-O157 STEC strains as human pathogens is underestimated (130) and 3) any outbreak or increase in incidence of STEC O157 will be significant for public health since its disease burden per primary case is estimated to be 12-fold higher than, for example, that for *Campylobacter* (respectively 55 and 4.6 Disability Adjusted Life Years per 1000 cases, which is the sum of potential life years lost due to premature mortality and the years of productive life lost due to disability) (59).

1.4 Sources of infection

Although cattle are considered to be the main reservoir for STEC and *Salmonella* Typhimurium, it should be realized that these pathogens can cycle through the environment and food chain via manure, insects, water, soil and food (fig. 2). Indeed, STEC was detected from various on-farm sources like water, feed and bedding material (42, 57) and from environmental sources like wild birds and rodents (95), insects (3), water (108) and soil (82). In the United States, between 1998 and 2005, a majority of 58% of the STEC outbreaks (n=342) were related to contaminated food and mainly occurred in the period from May to October (84). Partially in contrast with this, the most prominent risk factor for STEC cases in the Netherlands was contact with farm animals and manure (21%), followed by person to person spread (18%) and the consumption of raw or undercooked beef (12%) (130). During September and October 2005 the first national food related *E. coli* O157 outbreak occurred in the Netherlands, which was most likely caused by the consumption of contaminated steak tartar (45).

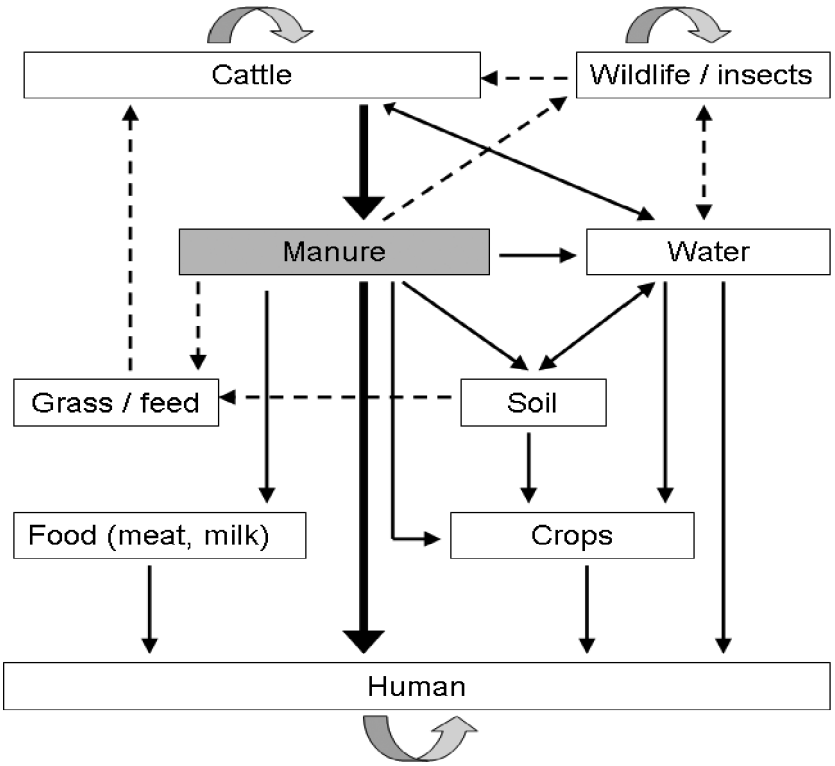

Fig. 2. Reservoirs and modes of transmission of STEC. Solid lines represent direct or indirect transmission routes between cattle and humans, dashed lines represent transmission lines back to cattle.

1.4.1 The role of fresh produce in the United States

During recent years a growing number food-borne illnesses has been associated with the consumption of fresh produce (114, 120). The number of reported produce-related outbreaks of food-borne diseases in the United States increased from two per year in the 1970s to seven per year in 1980s and to 16 per year in 1990s, while the total number of foodborne outbreaks with a known food item remained essentially constant (114). In addition, the median number of ill persons per produce-associated outbreak increased two-fold between 1973 and 1997, from 21 to 43. More recent data (1990-2004) even showed that produce caused the second highest number of foodborne disease outbreaks and highest number of disease cases among five major food categories (produce, beef, poultry, seafood and eggs) (11) (fig. 3). Seafood had the highest proportion of outbreaks (33%), followed by produce (22%), poultry (18%), beef (16%) and eggs (13%). However, produce accounted for the highest proportion of disease cases (38%), while seafood accounted for the lowest proportion (12%). Produce also showed the highest number of cases per outbreak (49 compared to 10, 30, 28, and 32 for respectively seafood, poultry, beef and eggs.

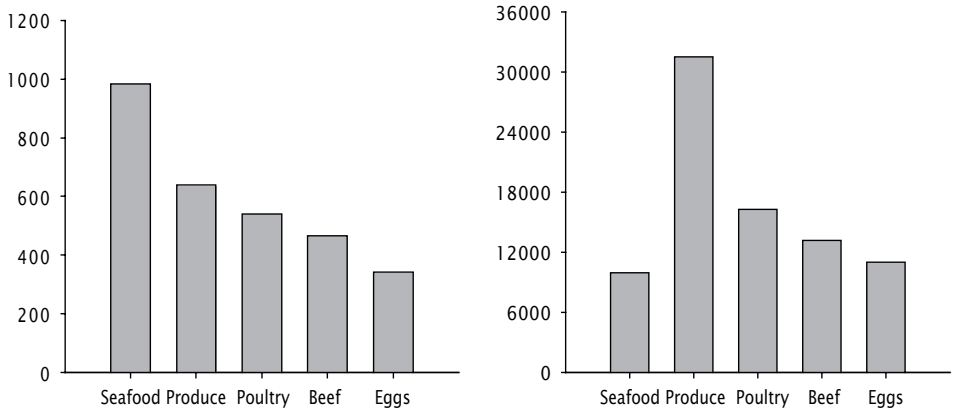

Fig. 3. Most common single-food vehicles linked to outbreaks in the US from 1990 to 2004 (data from http://www.cspinet.org/foodsafety/outbreak_alert.pdf).

Produce-associated outbreaks occurred throughout the year, with a peak in spring and a smaller peak in autumn. Among the produce-associated outbreaks, the food items most frequently implicated included salad, lettuce, juice, melon and sprouts (114) (fig. 4). Various pathogens have been implicated in produce-related outbreaks, with *Salmonella* and *E. coli* O157:H7 being the most dominant. Several food-pathogen combinations were reported, most commonly lettuce or fruit juice with *E. coli* O157:H7 and melon,

sprouts (i.e. seedlings of e.g. radish), or tomato with *Salmonella*. With respect to STEC, 30% (22% vegetables and 8% fruits) of all single food *E. coli* O157 outbreaks in the United States between 1998 and 2005 were produce related and 64% was cattle (dairy and beef) related (84). Notably, the largest outbreak of *E. coli* O157:H7 reported so far included approximately 6000 Japanese schoolchildren and was associated with the consumption of radish sprouts (87). Recently, a large multi-state outbreak of *E. coli* O157:H7 due to the consumption of contaminated fresh spinach in the USA received a lot of attention, as it resulted in 187 cases of illness, including 97 hospitalizations (which is 52%!) and three deaths (1.6%) (15). These recent outbreaks of *E. coli* O157:H7 and *Salmonella* infections associated with fresh produce in the USA indicate the increased importance of improving the microbial safety within the vegetable industry.

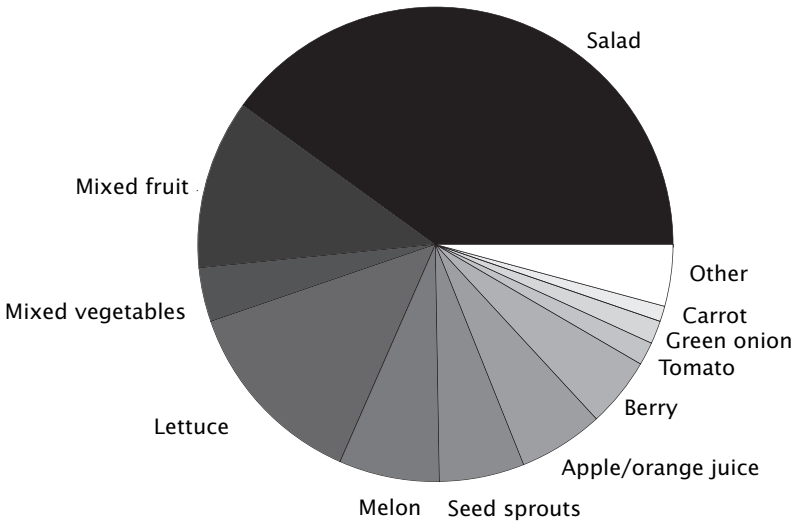

Fig. 4. Type of produce item associated with produce-related outbreaks of foodborne disease in the United States from 1973-1997 (n=99). Data from Sivapalasingam *et al.* 2004 (114)

1.4.2 The role of fresh produce in Europe

The European Union yearly produces about 50 million tons of vegetables and is the world's main importer of fruits and vegetables (13). The consumption of vegetables in 1998 was approximately 35 million tons, which is on average 94 kg/person/year. Fewer data on outbreaks are available for the EU compared to the US because it is only since 2005 that the reporting of foodborne disease outbreaks is mandatory for European Union member states. Since 2003 the rapid alert system of the European Union (http://ec.europa.eu/food/food/rapidalert/index_en.htm) publishes weekly

reports of food safety alerts and summarizes these reports in annual reports. An analysis of the Rapid Alert database gives some idea on the microbial problems with fresh produce (Table 1). From the annual reports it can be deduced that there is an increase in the absolute number of alert notifications for fruits and vegetables (fig. 5). However, this increase is parallel to the increase of total alerts; the relative proportion of alert notifications for fruits and vegetables remained fairly stable during the period 2000-2005 (approximately 9%). In 2005, 332 notifications were reported with respect to fruits and vegetables of which 38 (11%) implicated pathogenic micro-organisms.

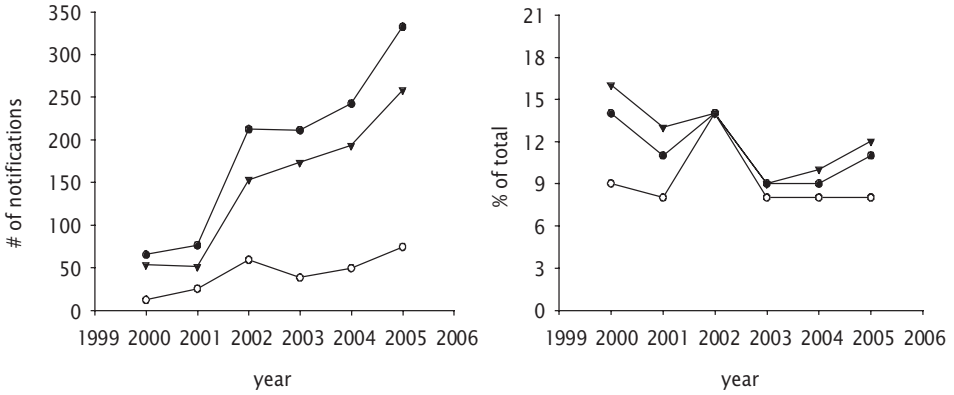

Fig. 5. Absolute number (left) and relative proportion (right) of total notifications (•), non-alert notifications (▼) and alert notifications (○) per year for fruits and vegetables as reported by the Rapid Alert system of the European Union (http://ec.europa.eu/food/food/rapidalert/index_en.htm)

Between 1992 and 2000, 83 outbreaks (5.5%) of foodborne disease associated with the consumption of fresh produce were reported from England and Wales, with *Salmonella* being the most reported pathogen (81). During this period, 3438 people were affected, 69 were hospitalized and one person died. Three community scale outbreaks were associated with contaminated lettuce as source and these accounted for 501 cases (14.6%) (81). Several of these outbreaks were international, involving several European countries. This indicates that contamination of fresh produce can result in geographically spread outbreaks with potential serious public health consequences. Interestingly, the total number of foodborne outbreaks in England and Wales during the period 1992-1999 declined while the absolute and relative number of produce-associated outbreaks remained more or less constant (13). A more recent report indicated 135 (7.7%) produce-associated outbreaks in England and Wales between 1992 and 2003, with *Salmonella* accounting for 21% and *E. coli* O157 for 3% of the outbreaks (12). During surveys in Sweden and Ireland respectively 0.5% and 0.03% of the investigated vegetable samples were found positive for microbial contaminants.

Higher figures have been reported in some countries but it is difficult to compare the data directly since it is not always possible to separate product categories (13). Moreover, the lack of robust traceability and weaknesses in reporting systems for outbreaks in most countries constrains comprehensive evaluation of the role of fresh produce as a source of foodborne disease in Europe.

1.4.3 Crops as host for human pathogens

Early outbreaks of foodborne disease linked to fresh produce were initially thought to result from cross-contamination of produce with other food items more likely to be a source of enteric pathogens, like meat products (30). However, surveillance data support the epidemiological likelihood of possible contamination of fresh produce with human pathogens in the pre- or postharvest environment. Numerous pathogens have been isolated from a wide variety of fresh vegetables, although not all of these pathogens have been implicated in produce-related outbreaks (22, 58). Recently, *E. coli* O157:H7 was isolated from 4 out of 101 fresh vegetable samples (89). Since many human pathogens have an enteric source, it is generally thought that they may be unsuccessful as plant colonizers relative to the more adapted plant microbial communities. However, evidence is emerging that enteric pathogens, although not as fit in the plant habitat as native plant-associated bacteria, have the ability to grow and persist on crop plants (30, 58). The increase in produce-related outbreaks, surveys showing the presence of human pathogens on produce and experiments indicating the persistence on plant tissue are suggesting that plants might be more important as a secondary habitat for enteric pathogens than previously thought. This might have severe implications for public health.

1.5 Outbreaks of *E. coli* O157 and *Salmonella* associated with lettuce

From 1973 to 1997, lettuce was the main implicated produce item within the group of single produce items responsible for produce-associated outbreaks in the United States (25 out of 85 outbreaks) (114). These 25 outbreaks caused 2078 illnesses, 181 hospitalizations and 6 deaths. *E. coli* O157:H7 and lettuce were found to be strongly associated with each other: 29% (5 out of 17) of all lettuce-related outbreaks were due to *E. coli* O157:H7 and 38% (5 out of 13) of all *E. coli* O157:H7 outbreaks with fresh produce were associated with lettuce. *Salmonella* was implicated in 18% of the lettuce-associated outbreaks and 10% of the produce-related *Salmonella* outbreaks were associated with lettuce. The average number of ill persons per lettuce associated *E. coli* O157:H7 outbreak was more than double as high as for *Salmonella* outbreaks (50

compared to 21 persons) (114). However, In England and Wales 41% of the fresh produce outbreaks were associated with *Salmonella* (81). Several lettuce-associated outbreaks with *E. coli* O157:H7 and *Salmonella enterica* are described (Table 2).

Table 2. Examples of lettuce-associated outbreaks with *E. coli* O157:H7 and *Salmonella enterica* serovars.

Year	Pathogen	No. of ill persons	Location	Reference
1995	*E. coli* O157:H7	23	Canada (Ontario)	(8)
1995	*E. coli* O157:H7	92 (1 HUS)	USA (Montana)	(2)
1996	*E. coli* O157:H7	61 (3 HUS)	USA (multiple states)	(64)
1999	*E. coli* O157:H7	37	Sweden	(140)
2005	*E. coli* O157:H7	135	Sweden	(115)
2006	*E. coli* O157:H7	71	5 states, USA	(9)
2000	*S.* Typhimurium	181	Iceland	(39)
2000	*S.* Typhimurium	361	United Kingdom	(65)
2005	*S.* Newport	368	United Kingdom	(14)
2005	*S.* Typhimurium	57	Finland	(119)

Recently (November-December 2006), an *E. coli* O157:H7 outbreak occurred across 5 USA states. At least 71 people became ill after consuming shredded iceberg lettuce as an ingredient in a specific fast-food restaurant (9). Of the confirmed cases, 53 (75%) were hospitalized and 8 (11%) developed HUS.

1.6 Possible reasons for increased vegetable outbreaks

There are a number of possible reasons for the increase in produce-associated outbreaks over the last decades. Improved diagnostics and increased surveillance could lead to an increased identification but would lead to an overall increase in reported foodborne outbreaks (120). If general awareness of the potential role of produce in disease outbreaks had increased among epidemiologists, it could have caused an artificial increase in reported outbreaks. While this could partly explain the increased number of outbreaks, questions about produce consumption have been asked routinely for decades and have only recently identified a large number of outbreaks (120). Given these two observations, the increase in reported produce-associated outbreaks seems real and other reasons, like changes in consumer food preference, food production and food distribution must be considered. During the last decades the consumption of fresh fruits and vegetables increased, partly due to nutritional guidelines for health improvement. Moreover, there is a wider variety of fresh produce available and globalization of the food supply has made fresh produce available year round. The latter also implies dependence on the application of good farming practices at farms

originating from a multitude of countries, including effects of storage needed for long-distance transport. Additionally, the application of technologies such at cutting, slicing and shredding will remove the natural protective barriers of the intact plant and open the possibility for providing a suitable medium for the growth of pathogens. These technologies also increase the risk of cross-contamination.

1.7 Mechanisms of vegetable contamination

Vegetables can become contaminated at any point in the production chain: during growth, harvesting, processing, distribution and final preparation. Preharvest contamination of vegetables can occur directly or indirectly via animals, insects, water, soil, dirty equipment and human handling. However, the most important considerations are the application of manure or compost as fertilizer to fields where crops are grown and the faecal contamination of irrigation water.

Cattle are considered to be the primary but transient reservoir of STEC, carry the pathogens usually asymptomatic and shed them in their faeces. Animal manure is intensively used worldwide as a crop fertilizer, especially in areas where intensive livestock farming co-occur with arable farming. This is the case in the Netherlands where annually approximately 17-35 tons manure (mostly from cattle) per hectare is applied within the lettuce production sector. This includes both conventional and organic production. A proportion of this manure will contain human pathogenic bacteria which will have the potential to enter the food chain when they are applied to fields used for the production of fresh produce like lettuce.

The prevalence of STEC O157 in cattle varies considerably within and between countries, depending on season, sampling strategy, detection method and geographic location (1-87% positive herds and 0-68% positive animals within the herd) (109). In the Netherlands, average herd prevalence has been estimated to fluctuate between 0 and 16%, with an average of 7.2% and reaching occasionally levels up to 36% (110). Within herds, prevalence ranged between 0.8% and 22.4% (62) and between 0% and 29.5% (111). Concentrations of STEC O157 in STEC-positive manure varied widely between 10 CFU and 10^6 CFU g^{-1} (49, 144).

Contamination of vegetables grown in soils enriched with contaminated manure will largely depend on the survival capabilities of the pathogen in manure and manure-amended soils. The conditions for survival of enteric pathogens are considered to be unfavourable once excreted from the animal gut (122). However, pathogens like STEC O157 and *Salmonella enterica* are able to survive for extended periods (up to months) in manure (28, 76, 112, 138) and manure-amended soil (56, 90, 97) and can become associated with vegetables grown in soils enriched with contaminated manure (70, 71, 92, 116). After

harvest, enteric pathogens can survive and even grow on the plant surface, depending on temperature, water availability, level of tissue damage, available nutrients, and the nature of the plants native microflora (1, 17, 30, 38). The shredding and subsequent distribution of contaminated crops at processing plants may lead to a significant spread of pathogens. Much attention has been given to the development of package technology to prevent spoilage of fresh produce (especially pre-cut produce), like modified atmosphere packaging (MAP). Normally, the concentration of oxygen with MAP is low (1-5%) to reduce respiration of the produce and ethylene production. However, oxygen concentration in MAP often reaches levels below 1%, which might favour the growth and survival of certain facultative anaerobic human pathogens while suppressing the background flora which might play an integral role in the suppression of these pathogens (48)

1.8 Methods to control risk of contamination

1.8.1. A systems approach

It was noted that most of the produce-associated outbreaks of foodborne disease in Europe were associated with intact products grown in contact with soil/water (13). Fewer outbreaks have been associated with processed products. Prevention of preharvest contamination of vegetables is an essential part of a systems approach focused on the development and implementation of intervention strategies to achieve the delivery of microbiologically safe vegetables to the consumer (24). This systems approach to control the safety of fresh produce should encompass the entire primary vegetable production chain, which includes cattle as the origin of the problem. This is not only beneficial for the vegetable industry which uses the manure but also for the dairy and animal production sector in order to minimize on-farm pathogen cycling and between-farm transmissions. Indeed, in an effort to minimize the level of human pathogens at the source, research is increasingly directed towards the identification of specific farm management factors that may be linked to an increased presence and/or shedding of these pathogens.

The inclusion of organic and conventional farming systems in this research provides the opportunity to study a wide array of different management systems, exhibiting a variety of animal manure, soil and plant characteristics. This will enhance the identification of risk factors. Under organic management, traditional conservation-minded farming methods are combined with modern farming techniques but conventional inputs such as synthetic pesticides and fertilizers are excluded. Instead of synthetic inputs, compost and animal and green manures are used to build up soil fertility; pests are controlled naturally, crops are rotated, and both crops and livestock are diversified (101). Organic arable systems have been found to

differ considerably from conventional systems concerning various biotic and abiotic characteristics (85, 127) and have been associated with increased suppression of plant pathogens (125, 126).

1.8.2 Control strategies at dairy farms

Considerable effort has been undertaken to identify herd management practices that are associated with the presence of *E. coli* O157 and *Salmonella enterica* in cattle. For *E. coli* O157 this has been reviewed excellently several times (19, 117, 135). Summarizing, potential control strategies can be grouped into three categories (Table 3) (79). Exposure reduction strategies aim at reducing bovine exposure to *E. coli* O157, exclusion strategies aim at modifying the microenvironment of the gastro-intestinal tract of cattle to prevent pathogen establishment or cause pathogen displacement, and direct anti-pathogen strategies specifically target and kill pathogens (78). Many of the potential control strategies follow from risk factor analysis with prevalence and management data, gathered during surveys. The effects of some of these factors, like cattle diet, have been investigated experimentally but often give ambiguous results. Current knowledge does not allow for a clear association between certain farm practices and pathogen shedding/prevalence. This is mainly due to the variability in practices, experimental setups and the interactions with other factors that might have been overlooked (78). Research on the effects of various potential control measures requires large studies in order validate the effect in a range of conditions and geographical locations. Potential control strategies like antimicrobial compounds and bacterial-phage therapy might require regulatory approval and/or consumer acceptance. In addition, these strategies might prove to be unsustainable because of the risk of resistance and increased under antibiotic stress (18).

Table 3. Overview of possible control strategies to control *E. coli* O157 in cattle. Adapted from LeJeune et al. (78).

Control strategy	Factors included	Reference
Exposure reduction	Ensure feed hygiene	(67)
	Ensure water hygiene	(47)
	Ensure housing hygiene	(42)
	Minimal wildlife exposure	(40)
	Avoid high cattle density	(137)
Exclusion strategies	Feeding regime	(44, 61, 106)
	Probiotics	(143, 145)
Direct anti-pathogen	Antimicrobial compounds	(31, 88)
	Bacteriophage therapy	(113)
	Vaccination	(99, 128)

For *Salmonella* clear overviews on risk factors associated with increased or decreased risk of prevalence in cattle are lacking. Nevertheless, several studies have identified such factors which could also be placed within the control strategy groups as described above for *E. coli* O157. The surrounding farm environment could be an important source of *Salmonella* contamination (104, 139), increased herd size can be considered as a risk factor for increased prevalence (53, 72, 124, 139), calve management might play an important role (43, 96, 136) and feeding strategies are identified as risk factors (51, 83, 124). Interestingly, feeding or grazing roughage from fields where manure was applied in solid or liquid form and not ploughed under during the same growing season was associated with increased *Salmonella* prevalence in a study including 129 farms (52).

1.8.3 Control strategies for growers

The two major mechanisms of produce contamination during the primary production phase are the use of contaminated manure for fertilization and the use of contaminated irrigation water. In the United States the focus is primarily on irrigation water since conventional vegetable production primarily relies on the use of artificial fertilizer and especially the last large *E. coli* O157:H7 outbreak associated with spinach contributed to this approach. However, because organic fertilizer like animal manure is the primary source for fertilization in organic farming, microbial risks associated with manure use is at the centre of attention for organic vegetable production (10).

In the Netherlands, the use of manure can be considered the primary mechanism since large-scale irrigation is not applied and manure is used as the primary source for fertilization in organic as well as in conventional vegetable production. During and after harvest several control measures are suggested like field worker hygiene, field sanitation, equipment sanitation, the use of clean water and temperature management during transport and storage. However, contamination can occur before harvest when the crops are grown in contaminated soil. In the United States, the USDA organic certification program requires the composting of raw manure, the application to land used for a crop not intended for human consumption, or the incorporation of manure into the soil at least 90 days before harvesting an edible product that does not come into contact with the soil and at least 120 days before harvesting an edible product that does come into contact with the soil (10). Compost must have reached a temperature between 55 and 76°C for 3-15 days (depending on the composting system). In the UK a 6 months interval between manure application and harvest was recommended (94). In the Netherlands there are, except for environmental regulations with respect to nitrogen leaching and emission, no rules for the application of animal manure to fields used for the production of vegetables in order to minimize the risk of contamination with human pathogens.

1.8.4 Sanitation

In response to the current public health concerns on the microbiological safety of fresh produce, research has focused on the efficiency of numerous physical, chemical and biological methods for reducing the (potential) pathogen load of produce during post-harvest processing. Chlorine is probably the most widely used sanitizer in the fresh-produce industry. However, chlorine concentrations traditionally used with produce (<200 ppm) are not particularly effective at reducing pathogen load on lettuce and using a higher concentration is not necessarily more effective (17, 48). Chlorine levels higher than 200 ppm are also thought to cause adverse decoloration (bleaching) and changed flavour of the produce (66). Other methods used include chlorine dioxide, bromine, iodine, alkaline compounds, quaternary ammonium compounds, organic acids, hydrogen peroxide, ozone and irradiation (23). Their effectiveness depends on the type of produce, characteristics of the produce surface, contact time etc. (48). The various sanitation procedures may not be sufficient in removing strongly attached bacteria on cut surfaces, pathogens may be protected against removal in biofilms and within tissue structures, while internalized pathogens might not be removed or killed at all. Irradiation might be sufficient but is relatively expensive. This might especially be a problem for pathogens with a very low infectious dose like STEC O157. In addition, the various decontamination methods also remove and kill a part of the native microflora, which might act as a barrier to the spread and growth of pathogens by competing for space and nutrients and/or the production of antagonistic compounds. Moreover, the naturally occurring microflora might have beneficial health effects like the stimulation of the immune system. Finally, the legal use of these different sanitizing procedures varies between countries and is mostly forbidden in organic production.

1.9 Quantitative Microbial Risk Assessment

A full risk analysis process consists of 3 interacting elements: *risk management*, *risk assessment* and *risk communication*. The risk assessment part constitutes the scientific part of the risk analysis and embraces four elements: *hazard identification, hazard characterization, exposure assessment* and *risk characterization*. The *hazard identification* identifies the issues of concern and provides the focus of the risk assessment. The *hazard characterization* part deals with the evaluation of the adverse health effects associated with a particular pathogen, which generally encompasses the establishment of a dose-response curve. The *exposure assessment* provides an estimate of how likely it is that an individual or a population will be exposed to a microbial hazard and what numbers of organisms are likely to be ingested (77). Finally, the *hazard characterization* and the *exposure assessment* are combined in the *risk*

characterization part to obtain a final estimate of risk (usually the likelihood and severity of the number of people in a population experiencing adverse health effects as result of the consumption of the contaminated food item of concern).

Quantitative microbial risk assessment (QMRA) modelling is increasingly used as a tool to evaluate food-related health risks (20, 36, 80, 93, 123). The CODEX Alimentarius Commission guidelines for the development of a microbial risk analysis gives a set of principles and definitions, but does not present a modelling methodology (6). Cassin *et al.* (36) introduced the process risk model (PRM) which integrates QMRA methodology with scenario analysis and predictive microbiology. The emphasis of the PRM is to apply QMRA as a tool for identifying intervention strategies that might mitigate risk and not the quantitative estimate of risk per se. For the exposure assessment the transmission of the microbial hazard is modeled through the food pathway, which is the chain of successive processes from a source to the moment of consumption. A probabilistic transmission model like the PRM follows probability distributions of the prevalence and the concentration of the hazard along the consecutive parts of the food pathway (for example production, processing, distribution and consumption), taking into account the variability attending this transmission. A probability distribution of the final risk can be obtained by performing so-called Monte Carlo simulations. This numerical technique is based on randomly selecting a single "point-estimate" value from each of the probability distributions assigned for each input parameter. These randomly selected values are used to calculate a mathematical solution defined by the risk assessment model, and the result is stored. This sequence is repeated many times (several thousands) with a different set of input values at each iteration. Values that are more likely to occur (according to the defined probability distributions of the input parameters) are selected more frequently. The outputs are combined into a probability distribution of the final risk reflecting the combined ranges and frequencies of the input parameters. Because risk is a chance of an effect to occur, stochastic modeling seems more appropriate to use in quantitative risk analysis.

1.10 Outline of this thesis

Prevention of preharvest contamination of fresh produce is an essential part of a systems approach focused on applying interventions designed to achieve delivery of microbiologically safe produce to consumers. Apart from some manure application regulations and recommendation, no intervention strategies are available at the moment to minimize the risk of contamination in the primary production phase of vegetables.

In order to minimize the risk of vegetable contamination, the survival and spread of human pathogens in the primary production chain should be minimized. This must

be realized by intrinsic factors of the manure and soil system, which can be altered by changes in farm management. The **two main objectives of this thesis** are 1) to identify risk factors for the survival and spread of *E. coli* O157:H7 and/or *Salmonella enterica* serovar Typhimurium in manure and manure-amended soil, 2) to construct a quantitative microbial exposure assessment model that estimates the probability of contaminated lettuce crops and the likelihood of the associated concentrations. An essential part of this thesis is the comparison of organic and conventional farming systems with respect to pathogen spread and survival.

Although under debate (33), the adjustment of cattle diets in order to reduce the prevalence and shedding of *E. coli* O157:H7 and *Salmonella* Typhimurium has been subject to several experimental and epidemiological studies. The effect of cattle diet on the subsequent survival of these human pathogens in manure is highly relevant within the framework of vegetable safety but rarely, if ever, investigated. **Chapter 2** investigates the difference in survival of *E. coli* O157:H7 and two morphotypes of *Salmonella* Typhimurium in manure resulting from dairy cattle experimentally fed three different roughage types and two levels of additional nitrogen concentrates. Chemical characteristics of the manure most contributing to the difference in survival are identified. In addition, survival of these pathogens was studied during several niche transitions: from manure to manure-amended soil and to manure-amended soil planted with lettuce. Finally, also lettuce is checked for the presence of both pathogens.

Although internalization of human pathogens into lettuce seedlings has been demonstrated recently (116), the quantification of this endophytic presence has not been subject of research so far. In **Chapter 3** the epiphytic and endophytic presence of *E. coli* O157:H7 and two morphotypes of *Salmonella* Typhimurium in lettuce seedlings, grown in soil amended with contaminated manure, is quantified.

Chapter 4 combines research on the natural prevalence of *E. coli* O157 and STEC virulence genes in dairy manure and the survival characteristics of *E. coli* O157:H7 in different manures. The prevalence of *E. coli* O157 and the STEC virulence genes are determined in manure samples from 16 organic and 9 low-input conventional dairy farms by real-time Taqman PCR. Subsequently, these 25 manures are inoculated with *E. coli* O157:H7 to study the variability in survival. An array of biotic and abiotic characteristics of the manures is determined and those factors most contributing to differences in pathogen presence and survival are determined.

An important part concerning the ecology of human pathogens in the primary lettuce production chain is the soil. In **Chapter 5** the effects of soil type and management type on the survival of *E. coli* O157:H7 in manure-amended soil is studied. Eighteen pairs of neighbouring organically and conventionally managed soils are collected and amended with inoculated manure in the laboratory. Biotic and/or abiotic factors most contributing to the variability in survival are identified with multivariate statistics.

Chapter 6 presents a probabilistic quantitative microbial exposure assessment model in which the exposure of the Dutch population to *E. coli* O157:H7 contaminated lettuce crops, grown in soils amended with cattle manure, is assessed. Only the transmission within the primary production chain is assessed (cattle-manure-soil-lettuce) and the farm-gate is regarded as the end-point of the exposure model. Post-harvest processes like transport, cutting, mixing and packaging are not modelled.

Finally the results obtained in this thesis research are discussed in **Chapter 7** in view of their contribution to scientific insights and practical risk assessment and development of a hazard analysis and identification of critical control points (HACCP).

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Chapter 2

The effect of cattle feeding regime and soil management type on the fate of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in manure, manure-amended soil and lettuce.

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Abstract

Survival of the green fluorescent protein-transformed human pathogens *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium was studied in a laboratory-simulated lettuce production chain. Dairy cows were fed 3 different roughage types: high digestible grass silage + maize silage (6:4), low digestible grass silage and straw. Each was adjusted with supplemental concentrates to a high and low crude protein level. The pathogens were added to manure which was subsequently mixed (after 56 and 28 d for resp. *E. coli* O157:H7 and *Salmonella* serovar Typhimurium) with 2 pairs of organically and conventionally managed loamy and sandy soil. After another 14 d, iceberg-lettuce seedlings were planted and checked for pathogens after 21 d of growth. Survival data were fitted to a logistic decline function (exponential for *E. coli* O157:H7 in soil). Roughage type significantly influenced the decline rate of *E. coli* O157:H7 in manure with the fastest decline in manure from the pure straw diet and the slowest in manure from the grass-silage + maize-silage diet. Roughage type showed no effect on the rate of decline of *Salmonella* serovar Typhimurium, although decline was significantly faster in the manure derived from straw compared to the manure from the grass-silage + maize-silage diet. The pH and fiber content of the manure were significant explanatory factors and were positively correlated with the rate of decline. With *E. coli* O157:H7 there was a trend of faster decline in organic compared to conventional soils. No pathogens were detected in the edible lettuce parts. The results indicate that cattle diet and soil management are important factors with respect to the survival of human pathogens in the environment.

2.1 Introduction

Agricultural animals are widely recognized as reservoirs of human enteric pathogens (30, 43). These pathogens are shed in their feces, which in turn could serve as the primary source for contamination of various food products. Most cases of human infection by these pathogens have been primarily linked to the consumption of animal food products. However, various pathogens have been recovered from vegetables (3) and the number of documented disease cases associated with the consumption of raw vegetables has increased in recent years (39, 43). Outbreaks of *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium have been associated with the consumption of lettuce (17, 19). Both human enteric pathogens have a principal reservoir in cattle (9, 53).

One possible mechanism of vegetable contamination with these pathogens is the land application of manure as fertilizer (32). The conditions for survival of enteric human pathogens are generally considered to be unfavorable once they are excreted from the animal (46). Possible contamination of vegetables grown in soil enriched with manure will largely depend on the survival capabilities of the pathogen in manure, in soil and in/on plants. Differences in animal feeding regime and the absence of synthetic fertilizers, pesticides and routine use of antibiotics may lead to differences in pathogen prevalence and survival between organic and conventional farming systems. Because animal manure is the major source of fertilization in organic crop production microbial safety is at the center of attention for organic vegetable production (2). However, it has not been demonstrated that the risk of contamination of fresh vegetables is higher with organic than with conventional production (29).

Diet composition, abrupt changes in diet or fasting may influence the shedding of *E. coli* O157:H7 (43). There has been considerable debate concerning the effect of hay feeding versus grain feeding on the shedding and acid resistance of *E. coli* O157:H7. Grain feeding can create a more acidic environment in the gut of cattle which leads to the selection for acid-resistant generic *E. coli*, which may include the considerably acid-resistant *E. coli* O157:H7 (11, 38). This dietary effect on shedding of *E. coli* O157:H7 is supported by some epidemiological data (16, 37) but other results point in another direction (39). The hypothesis has also been supported (45) or challenged by experiments conducted with ruminants inoculated with *E. coli* O157:H7 (15, 20, 25, 48). Besides affecting the shedding of pathogens, the feeding regime of cattle can be expected to affect manure composition and might thereby also affect pathogen survival capabilities in manure.

In bovine manure, *E. coli* O157: H7 is documented to survive for extended periods of time (6, 26, 28, 55). Also *Salmonella* serovar Typhimurium is capable of survival for considerable periods of time in manure (18) and slurry (18, 24). Survival of excreted pathogens in freshly produced manure will be affected by the manure management system used on the farm: manure is handled as slurry or as solid manure, applied to fields after

a range of storage times and applied by surface spreading or injection into the soil (31). So far, the potential influence of cattle diet on pathogen survival in manure has not been subject of research. In manure-amended soil, reported survival times of *E. coli* O157:H7 vary considerably, from several weeks (32) to several months (6, 21, 23, 28). Long term survival has also been demonstrated for *Salmonella* serovar Typhimurium (22, 30).

E. coli O157: H7 and *Salmonella* may be transferred from manure-amended soil or manure compost-amended soil to leaf and root vegetables and can persist for long periods of time on these vegetables (21, 22, 30). Recently it has been shown that *E. coli* O157: H7 can become internalized in lettuce by entering the plant through the root system from a planting mixture of manure and soil and migrate throughout the edible part of the plant (41). Because of the lack of chemical treatments for controlling pathogen invasion in lettuce production, suppression of pathogens must rely solely on the antagonistic capacity of the resident microflora in the different econiches. Functional and taxonomic diversity and biomass of soil microbial and faunal communities are frequently higher in organic than conventional fields and have been correlated to a higher suppression of soil-borne plant pathogens (50, 51).

At present there is insufficient information about the influence of cattle diet and manure characteristics on the survival of human pathogens in manure. It is also not known whether organically and conventionally managed soils differ in the capability to suppress human pathogens. Moreover, the possible internalization of human pathogens in the edible parts of leafy vegetables grown in manure amended soil is scarcely documented. Previous studies on pathogen survival in the agricultural environment primarily focused on single parts of the lettuce production chain, like manure, soil or manure-amended soil with crops. In the present study, we simulated the lettuce production chain in the laboratory and followed the fate of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in three subsequent niches: manure, manure-amended soil and plant. The objectives of the present study were to determine pathogen survival as function of cattle diet, soil type and soil management (organic or conventional). Furthermore, the possibility of (internal) contamination of lettuce after a period of pathogen survival in manure and manure-amended was investigated.

2.2 Materials and Methods

2.2.1 Bacteria

Strain *Escherichia coli* O157:H7 B6-914 *gfp*-91 was kindly provided by Dr. Pina Fratamico (13). This strain does not produce the Shiga-like toxins I or II ($Stx1^-$ $Stx2^-$), but contains the pGFP cDNA vector (Clontech Laboratories, Inc. Palo Alto, CA) expressing green

fluorescent protein (GFP) and ampicillin resistance. The survival characteristics of the GFP-labeled strain were indistinguishable from those of the wild-type strain (13). In addition, Kudva (26) reported no differences in survival in bovine manure and manure slurry between toxin positive ($Stx1^+$ $Stx2^+$) and toxin negative ($Stx1^-$ $Stx2^-$) *E. coli* O157:H7. Two phenotypes of *Salmonella enterica* serovar Typhimurium, MAE 110 (Pagf D1, rdar: aggregate phenotype) and MAE 119 (ΔagfD101, saw: wild type morphology), were kindly provided by Dr. Ute Römling (35, 36). Both strains were derived from strains MAE 51 and MAE 52 respectively and both contain kanamycin and gentamycin resistance and the GFP gene on the chromosome after transformation with the PAG408 mini-transposon (42). The two strains can be distinguished by their appearance under UV-light. The colony shape of MAE 110 is larger, flatter, more ragged and less bright compared to MAE 119. Bacteria were stored at –80°C and checked for viability prior to use.

2.2.2 Cattle feeding and manure collection

Manure was obtained from an ongoing experiment on the effect of diet on manure quality by the department of Animal Science of the Wageningen University and Research Centre, the Netherlands (J.W. Reijs, personal communication). Dairy cows (Holstein Frisian, 3-7 years of age) were housed in one stable under identical conditions. Six couples (n = 2) of animals were fed six different diets for nearly 9 weeks (from 20 January 2003 until 21 March 2003): high-digestible grass-silage (40%) + maize-silage (60%) (GM), low-digestible grass silage (GO) and straw (S), each adjusted with supplemental concentrates to a high (H) and low (L) crude protein level (Table 1). Fresh manure (without urine) was collected directly from the couples of cows (with equal amounts of manure from each individual well mixed in a bucket) at the end of the feeding trial (after 9 weeks) and stored at 5°C in 20-liter containers.

2.2.3 Soils

An organically and a conventionally managed sandy soil, cropped to potatoes, were collected from two neighboring farms in Marknesse (Flevoland, the Netherlands). Organic and conventional loam soils, cropped to onions, were collected from two neighboring farms in Ens (Flevoland, the Netherlands). Both organic farms were SKAL-accredited (inspection body for organic production in the Netherlands) and thus refrained from the use of artificial fertilizers or pesticides. However, both organic and conventional farmers used animal manures as fertilizer. Throughout each field, 15 soil samples (1-20 cm deep) were collected between the plants with an augur and mixed. Samples were transported in plastic bags to the lab, stored at 5°C and sieved (4 mm) before use.

2.2.4 Inoculation of manure

A simulation of the transitions in the lettuce production chain from manure to soil and plants was done separately for *E. coli* O157:H7 and for a mixture of both *Salmonella* serovar Typhimurium phenotypes. Inoculum was prepared in Luria-Bertani broth with 50 µg/ml ampicillin for *E. coli* O157:H7 B6-914 gfp-91 and 50 µg/ml kanamycin for the *Salmonella* serovar Typhimurium phenotypes. Both phenotypes were grown separately and mixed to equal amounts before inoculation of the manure. Cells were harvested by centrifugation at 3000 × g (Hermle 2384 K), washed with and resuspended in 0.1% peptone buffer (Oxoid) to a density of 1 × 109 colony forming units (CFU) per milliliter. This cell density was determined spectrophotometrically, taking into account that an optical density (OD)630 of 1 would equal approximately 0.7 × 109 CFU ml-1. The dry-weight of the manure was determined by drying overnight at 105°C. Cells were added to a final density of 1 × 107 CFU per gram manure dry weight (gdw-1). For *Salmonella* serovar Typhimurium a mixture of 0.5 × 107 CFU MAE 110 gdw-1 plus 0.5 × 107 CFU MAE 119 gdw-1 was added to manure. After mixing, by thoroughly kneading the manure in a plastic bag from the outside by hand, 500 gram of the inoculated manure was transferred to a pre-weighed plastic pot (1 L) which was closed but with the ability of gas exchange. There were 3 replicate pots per manure type and the same amount of non-inoculated pots, which functioned as blanks, with 0.1% peptone buffer added instead of bacterial suspension. The pots were weighed and incubated at 10°C in darkness. At each sampling time, pots were weighed before and after sampling to check for evaporation. Moisture content remained constant (on average around 85%) during the experiment. In addition, at each sampling time manure samples from the blanks were dried overnight at 105°C to determine their dry-weight.

2.2.5 Plate counts of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium

The inoculated pots were sampled over time to determine the survival of the pathogens in manure (at t=0 and after 3, 8, 16, 22, 28, 43, 56, 84 and 133 days). At each sampling time two samples of approximately 1 g of each replica was removed from the middle of each mixture using a sterile spoon and put in separate pre-weighed dilution tubes with 4.5 ml of 0.1% peptone. Sampling holes were closed. Sample-containing tubes were weighed to determine the exact size of the sample. Samples were vortexed and put in a ultrasonic bath for 30 s (Branson 5200, 120W output power, 47 kHz). The samples were vortexed again and 10-fold serial dilutions were made. From the two highest dilutions 50 µl was plated in duplicate on Petri dishes with Sorbitol MacConkey

Table 1. Description of the six types of diet fed to dairy cows in an experimental, controlled set-up (J.W. Reijs, personal communication).

Manure type	Roughage	Concentrates	CP[1]	VEM[2]	NDF[3]	ADF[4]
GMH	60% high digestible grass silage & 40% maize silage	40% soya & 60% maize	180 (H)	971	370	232
GOH	100% low digestible grass silage	100% soya	176 (H)	799	461	320
SH	100% straw	75% soya & 25% maize	185 (H)	772	504	334
GML	60% high digestible grass silage & 40% maize silage	55% maize & 45% beet pulp	116 (L)	970	392	246
GOL	100% low digestible grass silage	19% soya & 81% beet pulp	104 (L)	772	524	349
SL	100% straw	21% maize & 58% beet pulp	105 (L)	761	565	357

[1] Crude protein level of the total diet (g kg^{-1} dw); CP = % N x 6.25; H=high, L=low

[2] Energy level of total diet (kg^{-1} dw), 1 VEM = 6.904 kJ net energy.

[3] Neutral Detergent Fiber of total diet: cellulose, hemi-cellulose and lignin (g kg^{-1} dw).

[4] Acid Detergent Fiber of total diet: cellulose + lignin (g kg^{-1} dw).

Table 2. Chemical characteristics of six types of cattle manure, collected directly from cows fed on six diets as described in Table 1.

Manure	pH	N-NH_4	dry matter	N total	C total	C/N	NDF[1]	ADF[2]
		mg/kg	g/kg	g/kg	g/kg		(%)	(%)
GMH	6.1	364.65	123.32	32.76	463.17	14.14	49.41	34.16
GOH	6.9	282.42	186.09	21.33	382.75	17.94	53.28	37.07
SH	7.0	33.12	114.11	13.57	454.45	33.49	66.45	50.32
GML	6.4	255.39	137.64	30.27	467.89	15.46	46.62	34.07
GOL	7.0	122.74	153.88	19.67	409.16	20.80	55.16	39.33
SL	7.8	48.49	137.46	19.04	341.29	17.93	64.43	46.82

[1] Neutral Detergent Fiber: cellulose, hemi-cellulose and lignin in organic matter.

[2] Acid Detergent Fiber: cellulose + lignin in organic matter.

(SMAC, Oxoid) agar with ampicillin (50 μg/ml) for the enumeration of *E. coli* O157:H7 or on Luria-Bertani medium with kanamycin (50 μg/ml) for the enumeration of *Salmonella* serovar Typhimurium. The number of necessary dilutions was estimated based on preliminary counts. This resulted in 2 plates per dilution, 4 plates per sample and thus 8 plates per replica. When low cell numbers were expected, 16 or 32 plates per sample were used to increase the detection limit. Cell suspensions were spread on the surface by shaking with 2 mm sterile glass beads. The inoculated plates were sealed with parafilm and incubated at 37°C for 24 hours. Numbers of *E. coli* O157:H7 and of *Salmonella* serovar Typhimurium were determined by counting green fluorescent CFU using a dark-blue lamp (Philips PL-S 9W/08 Blacklight Blue, peak at 365 nm UV-A). Colony shape and gfp-intensity enabled distinction between *Salmonella* serovar Typhimurium phenotypes 110 and 119. Colony counts were calculated to number of CFU gdw-1.

2.2.6 Transmission to and survival in soil

To determine survival in manure-amended soil a subset of 60 g fresh weight (gfw) of manure was mixed with 540 gfw of each of the four soils (1:9). These mixtures were mixed thoroughly in plastic bags by hand and transferred to similar plastic pots (1 L.) as used in the survival in manure part of the experiment. For *E. coli* O157:H7 this was done 56 d after inoculation with manure type GMH and GML because the other manure types showed too low numbers of pathogens for further transition to soil at that time. With the *Salmonella* serovar Typhimurium experiment pathogen levels allowed amending of the 4 soils with the two more contrasting manure types GMH and SH, which was done after 28 d of survival in manure. The pots were incubated at 15°C in darkness. For each manure-soil combination there were 3 replicate pots. Soils for the non-inoculated pots (blanks) were mixed in the same way with the manure blanks of the manure survival part of the experiment. Sampling of the inoculated pots to determine survival was done as described above (at t=0 and after 2, 7, 13, 28 and 57 d).

2.2.7 Lettuce production

Two weeks after mixing the manure with soil aliquots of 500 gfw mixture was transferred to plastic pots, in each pot one seedling of iceberg lettuce (*Lactuca sativa* L cv. Dublin) was planted (3 replicate pots x 8 treatments = 24 plants on inoculated soil mixture and 24 plants on non-inoculated blanks) and the pots were placed completely randomized on a greenhouse bench (15°C, RH 60%). After 3 weeks root samples (1-2 gfw) and shoot samples (on average 3 small leaves, 1-2 gfw) were checked for pathogen presence.

Root samples were washed in sterile water twice to remove soil particles. Both root and leaf samples were ground with pestle and mortar in 5 ml proteose-peptone (Oxoid) and crystal sand and plated (100 µl) directly on selective media as described earlier. To distinguish between epiphytic and endophytic pathogen presence, half of the samples were surface-sterilized for by dipping in 1% $AgNO_3$ for 10 s and washed 2 times in sterile water before grounding. Bulk soil samples were plated as described before.

2.2.9 Chemical measurements

Chemical characteristics were determined before starting the experiment for each manure (Table 2) and soil type (Table 3).

Manure. Dried samples (40°C) were ground and analyzed for total carbon by the Dumas method followed by detection by a CHN1110 element analyzer (CEInstruments, Milan, Italy) and for fiber content (52). Total nitrogen content was determined by the Kjeldahl method (5) and ammonium content was determined in a solution of trichloro-acetic-acid (TCA) by an Autoanalyzer II (Technicon Instrument Corporation, Tarreytown, NY). The pH was measured in a watery suspension with an Inlab pH level 1 (WTW GmbH, Weilheim, Germany).

Soil. Total nitrogen and carbon were determined as with manure. Nitrate and ammonium content in soil samples were determined with an Autoanalyzer II (Technicon Instrument Corporation, Tarreytown, NY) after addition of 0.01M $CaCl_2$ suspensions. The pH of the soil samples was measured in this $CaCl_2$ suspension with an Inlab pH level 1 (WTW GmbH, Weilheim, Germany).

2.2.10 Statistical analysis

Microbial data (CFU counts) were log transformed and these log numbers over time for each replica were fitted to the following logistic function by nonlinear regression (method = Gauss-Newton): $CFU(t) = a + (b / (1 + e^{(c - dt)}))$, where $CFU(t)$ is the log number of CFU gdw^{-1} on day t, a is the lower asymptote, $(a+b)$ is the upper asymptote, d is the slope of the regression curve at the steepest part of the curve (referred to as decline rate) and c is the lag-parameter determining the duration of lag-phase and thereby the location of the inflection point (referred to as lag-phase) (SAS version 8, SAS Institute, Cary, NC, USA). Since the slope parameter d is multiplied by time this parameter has far more weight in determining the shape of the curve. Time point zero was defined as the first sampling time, which occurred immediately after inoculation, the upper and the lower asymptote were kept constant at respectively 7 log CFU gdw^{-1} and 0. Time points which gave a CFU count of zero were included in the analysis with the value of 1 log CFU gdw^{-1} which was the detection limit. Significance of the fit was

assessed by a F-test ($F = MS_{regression} / MS_{residual}$) and the goodness-of-fit was determined by calculating a pseudo-R^2 ($1-[SS_{residuals}/SS_{total\ corrected}]$). For *E. coli* O157:H7, the number of days needed to reach the detection limit of 1 log CFU gdw^{-1} was calculated from the fitted decline function. Multivariate analysis of variance (MANOVA, significant level of 5%) followed by contrast analysis was conducted on the regression parameters *c* and *d*. From the second part of the MANOVA (within-subjects comparisons) the effects of roughage type and crude protein level on the decline rate (*d*) and lag-phase (*c*) were assessed. Differences in decline rate between *Salmonella* serovar Typhimurium phenotypes were analyzed by two-sided t-tests. Correlation tests were conducted to check for linear relationships between decline rate and chemical parameters of the manure. Stepwise multiple regressions were conducted to determine to what extent variation in chemical and biological parameters can explain variation in decline rates. Variables left in the regression model were significant at the 0.15 level and models were restricted to a maximum of 2 parameters.

Decline of *E. coli* O157:H7 in manure-amended soil was analyzed by fitting survival data (log CFU gdw–1) of each replica to a simple exponential decline function: CFU(t) = N0 Í est, where CFU(t) is the log number CFU gdw–1 on day t, N0 the initial log number CFU gdw–1 on day 0 and s is the slope of the curve. Because all treatments showed an increase during the first two days (see Results), the log number CFU gdw–1 on day 2 was set to 100%. The subsequent log numbers CFU gdw–1 were relative to day 2. Slopes of the different treatments were compared with two-sided t-tests. When no CFU were detected, the value of the detection limit was used (0.5 log number CFU gdw–1). Decline of both phenotypes of *Salmonella* serovar Typhimurium was analyzed by fitting the survival data to the same logistic function as done with the data of survival in manure because of bad fits (no convergence or low pseudo-R2) to the exponential model.

2.3. Results

2.3.1 Survival of *E. coli* O157:H7 in manure

In all manure types, *E. coli* O157:H7 populations dropped directly after inoculation by approximately 1.5 log CFU gdw^{-1}, followed by a period of around 16 days where it stabilized or increased by approximately 0.75 log CFU gdw^{-1} (GOL and SH) (Fig. 1). Thereafter, the pathogen declined continuously in all treatments. *E. coli* O157:H7 was not detected by plate counting anymore after 84 days in both manures derived from a straw diet (SH, SL) and after 133 in the other manure types.

Nonlinear logistic regression resulted in significant fits ($p<0.001$) with high goodness-of-fit values for all six manure types (average pseudo-R2 over 3 replica's: GMH: 0.92, GOH:

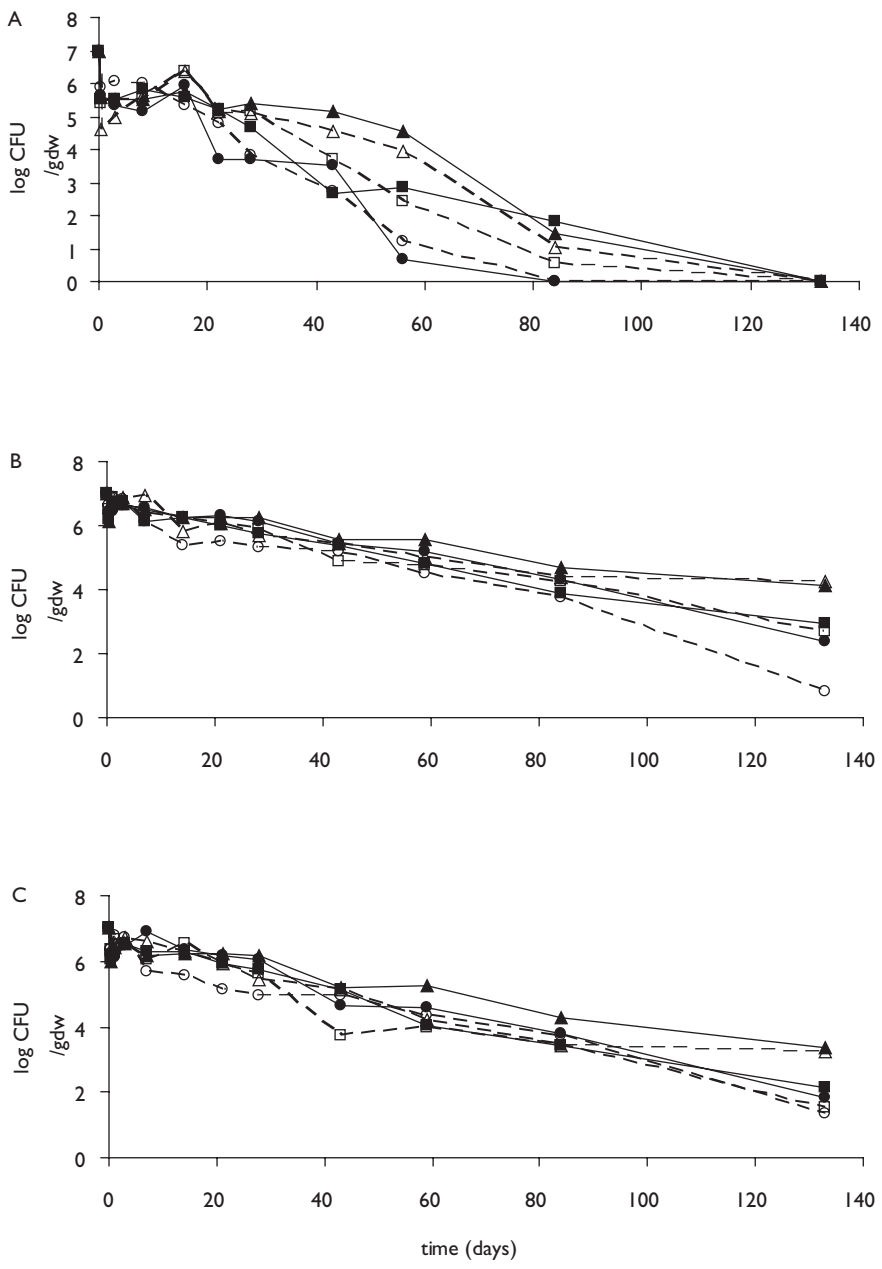

Fig.1. Survival of *E. coli* O157:H7 (A), *Salmonella* serovar Typhimurium MAE 110 (B) and *Salmonella* serovar Typhimurium MAE 119 (C) in six different types of artificially inoculated cattle manure types resulting from 3 different roughage types with high (closed symbols and solid lines) and low (open symbols and dashed) levels of additional crude protein: high digestible grass/maize silage (▲, Δ), low digestible grass silage (■, □) and straw (●, ○).

0.92, SH: 0.89, GML: 0.82, GOL: 0.93 and SL: 0.95). The number of days needed to reach the detection limit of 1 log CFU gdw-1 according to the logistic fits was for GMH, GOH, SH, GML, GOL and SL respectively 128 ± 8, 105 ± 8, 76 ± 5, 126 ± 18, 92 ± 12 and 71 ± 8. Roughage type had a significant effect (Wilks' Lambda =0.060, $p<0.001$) on the course of decline (effect on combined variance of both estimated parameters). Moreover, roughage type had a significant effect on the slope of decline ($p<0.001$) and crude protein level did not. Roughage type and crude protein level showed no interaction with respect to their effect on the decline rate. The lag-phase was not significantly influenced by roughage type or crude protein level. Decline rates in manures based on the same roughage type, but different crude protein levels, did not differ (Fig. 2). All three roughage types differed significantly from each other with respect to the slope of decline, irrespective of crude protein level. When the manure types from the high and low CP groups were aggregated to roughage type *E. coli* O157:H7 declined faster in manure derived from a diet of straw (S) compared to low-digestible grass-silage (GO) ($p=0.007$), S compared to high-digestible grass-silage + maize-silage (GM) ($p<0.001$) and GO compared to GM ($p=0.0272$).

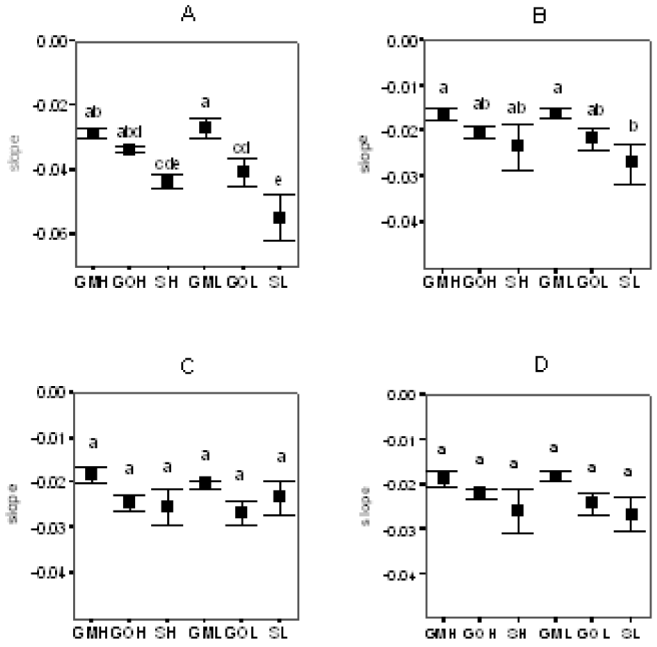

Fig.2. Values of the estimated slope parameter for the survival of *E. coli* O157:H7 (A), *Salmonella* serovar Typhimurium phenotype MAE 110 (B), *Salmonella* serovar Typhimurium phenotype MAE 119 (C) and *Salmonella* serovar Typhimurium total counts (D) in six different types of artificially inoculated cattle manure types resulting from 3 different roughage types with high (H) and low (L) levels of additional crude protein: high digestible grass/maize silage (GMH and GML), low digestible grass silage (GOH and GOL) and straw (SH and SL). Error bars show mean +/- 1.0 standard error of the mean. Treatments showing identical letters do not significantly differ.

Table 3. Physical and chemical characteristics of four soils, collected as neighboring pairs in the Netherlands, used for mixing with pathogen inoculated cattle manure which in turn functioned as a substrate for the growth of lettuce seedlings.

location	management	soil type	code	clay	silt	sand	pH	$N\text{-}NO_3$	$N\text{-}NH_4$	N-total	C-total	C/N
				%	%	%		mg/kg	mg/kg	mg/kg	mg/kg	
Marknesse	organic	sand	OS	3.2	33.3	63.5	7.1	20.92	29.69	2.28	23.78	10.43
Marknesse	conventional	sand	CS	3.2	32.4	64.5	7.1	5.20	30.18	1.35	14.45	10.70
Ens	organic	loam	OC	8.3	54.5	37.2	7.3	4.70	21.74	1.50	16.67	11.11
Ens	conventional	loam	CC	7.7	51.9	40.4	7.3	8.86	26.66	1.56	18.34	11.76

Table 4. Pearson correlation coefficients between the absolute slope values of the fitted logistic decline curve in manure and chemical characteristics of the six types of manure (N=18).

	Slope O157[1]	Slope 110[2]	Slope 119[3]	Slope Typh[4]	pH	N-total	C-total	$N\text{-}NH_4$	C:N	Dry Matter	NDF
pH	0.96*	0.97*	0.63	0.90*							
Ntotal	-0.13	0.00	0.33	0.16	0.13						
Ctotal	-0.72	-0.76	-0.45	-0.69	-0.86*	-0.52					
$N\text{-}NH_4$	-0.87*	-0.87*	-0.72	-0.87*	-0.81	0.27	0.39				
C:N	0.42	0.48	0.65	0.60	0.30	-0.36	0.13	-0.71			
Drymatter	-0.76	-0.84*	-0.90*	-0.94*	-0.75	-0.05	0.46	0.86*	-0.81*		
NDF[5]	0.89*	0.91*	0.61	0.85*	0.79	-0.29	-0.45	-0.88*	0.75	-0.88*	
ADF[6]	0.85*	0.86*	0.60	0.81*	0.74	-0.37	-0.35	-0.81*	0.81	-0.88*	0.99*

*significant correlation ($p<0.05$)

[1] *E. coli* O157:H7

[2] *Salmonella* serovar Typhimurium MAE 110

[3] *Salmonella* serovar Typhimurium MAE 119

[4] *Salmonella* serovar Typhimurium total counts

[5] Neutral Detergent Fiber: cellulose, hemi-cellulose and lignin in organic matter.

[6] Acid Detergent Fiber: cellulose + lignin in organic matter.

The rate of decline (absolute value of slope) was positively correlated with pH (p=0.003) and fiber content (Acid Detergent Fiber: p=0.032 and Neutral Detergent Fiber: p=0.017) (Table 4). The GM-manures had the lowest pH and lowest decline rates while the S-manures had the highest pH and the highest decline rate (Fig.3). The GO-manures had intermediate pH and intermediate decline rate. The rate of decline showed a negative linear relationship with ammonium level (p=0.024). Stepwise multiple regressions revealed that pH explained most of the variation in decline rate: slope [model R2=0.97] = -1.80 × 10-2 [pH; partial R2=0.91, p=0.003] + 1.06 × 10-4 [dry matter content; partial R2=0.06, p=0.056] + 7.03 × 10-2 [intercept]. Alternatively, when excluding pH, the neutral detergent fiber (NDF) content was best explaining the variation in decline rate: slope [model R2=0.93] = -2.19 × 10-3 [NDF; partial R2=0.80, p=0.016] + 7.05 × 10-4 [C:N ratio; partial R2=0.13, p=.093] – 3.35 × 10-3 [intercept]. The pH and NDF content were not significantly correlated (Table 4).

2.3.2 Survival of *Salmonella* serovar Typhimurium in manure

The two phenotypes MAE 110 and 119 showed rather similar survival curves and both survived clearly longer in all manure types than *E. coli* O157:H7 (Fig. 1). CFU counts dropped directly after inoculation with 0.5 log CFU gdw^{-1},followed by an increase of 0.5 log CFU gdw^{-1} within a few days. Thereafter, the pathogen declined continuously but at a lower rate than *E. coli* O157:H7. After 133 d *Salmonella* serovar Typhimurium could still be detected at levels of 2-4 log CFU gdw^{-1} by the normal plating procedure, depending on the manure type.

As with *E. coli* O157:H7, nonlinear logistic regression resulted in significant fits (p<0.001) with high goodness-of-fit values for phenotype MAE110 (average pseudo R2: GMH: 0.84, GOH: 0.84, SH: 0.89, GML: 0.71, GOL: 0.86, SL: 0.85) and MAE 119 (average pseudo R2: GMH: 0.90, GOH: 0.94, SH: 0.90, GML: 0.83, GOL: 0.86, SL: 0.87). *Salmonella* serovar Typhimurium MAE 110 and 119 showed no difference in slope over all treatments (p=0.223). Since both phenotypes behaved similarly, the effect of roughage type and crude protein (CP) level were assessed by summing up the CFU counts of phenotypes 110 and 119. There was a significant multivariate effect of roughage type (Wilks' Lambda=0.300, p=0.008) and CP level (Wilks' Lambda=0.516, p=0.026) on the combined variance of both regression parameters, but no significant effects of roughage type and CP level separately on the decline rate or lag-phase. Contrast analysis legitimated the pooling of manure types based on the same roughage type but different CP level (Fig. 2). When grouped by roughage type, *Salmonella* serovar Typhimurium declined significantly faster in the manure resulting from the straw (S) diet compared to the high-digestible grass-silage + maize-silage (GM) diet (p=0.020). The rate of decline was positively correlated with pH (p=0.017) and fiber content (NDF: p=0.005 and ADF: p=0.012) (Table 4 and

Fig. 3). The rate of decline showed a negative linear relationship with ammonium level (p=0.012) and dry matter content (p=0.010). Stepwise multiple regressions revealed that neutral detergent fiber (NDF) content explained most of the variation in decline rate: slope [model R2=0.97] = -2.97 × 10-4 [NDF; partial R2=0.91, p=0.003] – 2.46 × 10-3 [pH; partial R2=0.06, p=0.114] + 0.01081 [intercept]. Alternatively, when excluding neutral detergent fiber content and the parameters with which it was significantly correlated (ADF, dry matter content) (Table 4), the pH was best explaining the variation in decline rate: slope [model R2=0.95] = -4.98 × 10-3 [pH; partial R2=0.81, p=0.015] – 2.08 × 10-4 [C:N ratio; partial R2=0.14, p=0.056] + 1.56 × 10-2 [intercept]. The pH and NDF content were not significantly correlated (Table 4).

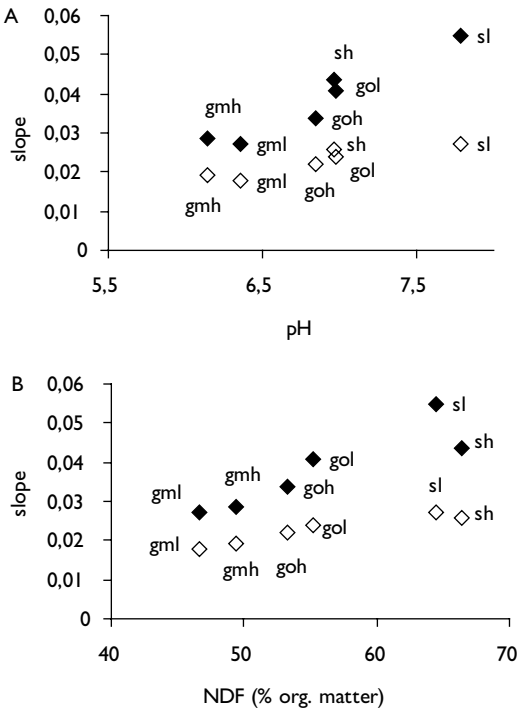

Fig. 3. Relation between pH (A) and NDF-fiber content (B) and the rates of decline of *E. coli* O157:H7 (closed symbols) and *Salmonella* serovar Typhimurium (closed symbols) in six different types of artificially inoculated cattle manure types resulting from 3 different roughage types with high (H) and low (L) levels of additional crude protein: high digestible grass/maize silage (GMH and GML), low digestible grass silage (GOH and GOL) and straw (SH and SL).

2.3.3 Survival of *E. coli* O157:H7 in soil

Survival of *E. coli* O157:H7 in the 4 soils amended with both manures derived from high-digestible grass-silage + maize-silage diets (GMH and GML), varied between 2 and 56 days, depending on the soil (Fig. 4). Fitting the survival data to an exponential decline function resulted in good fits (average R2 over all treatments of 0.87 0.17). The values of the estimated slope of decline are shown in Fig. 5. The kind of manure applied to the soil made

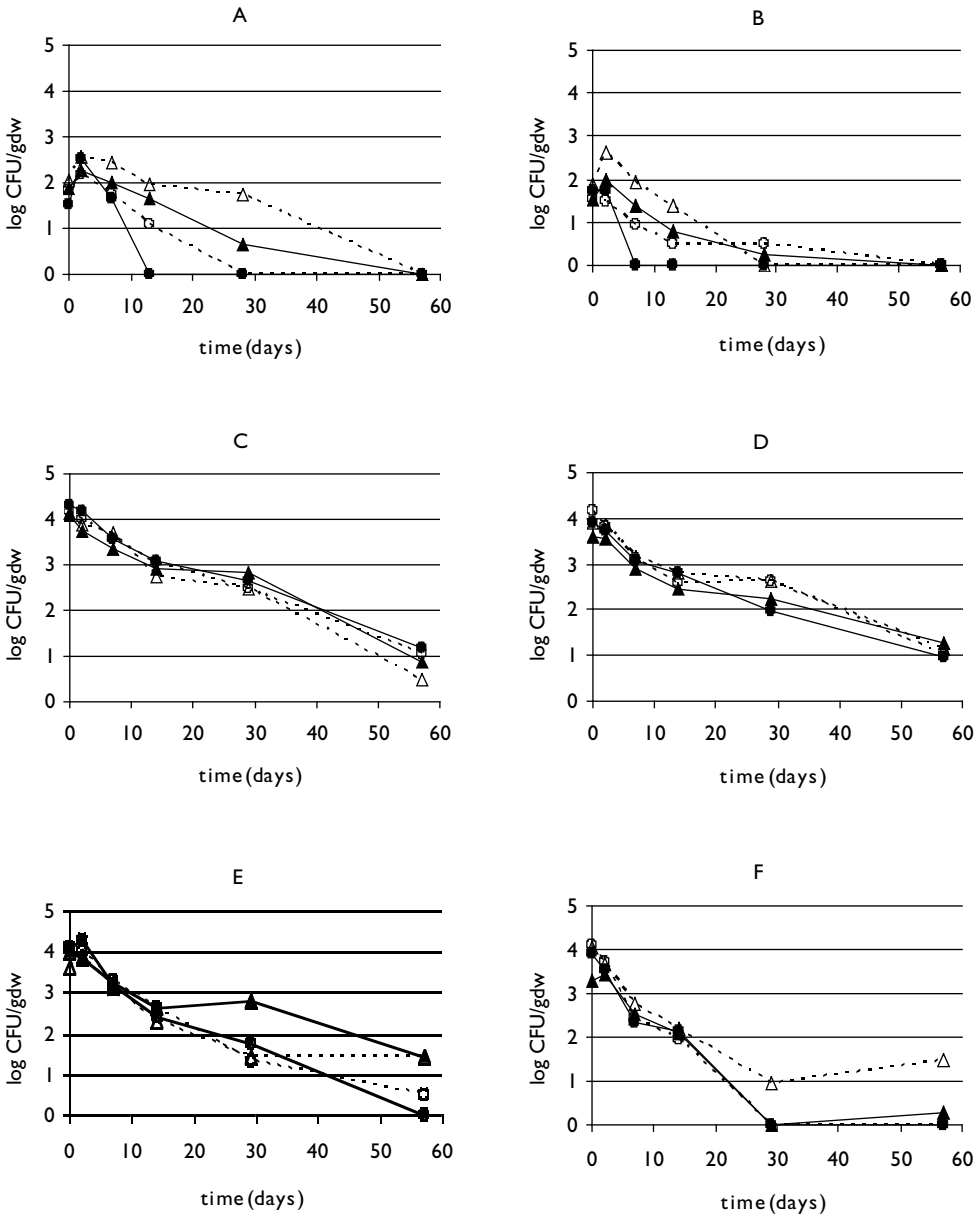

Fig. 4. Survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in 4 different soils: organic sand (● solid line), conventional sand (○ dashed line), organic loam (▲ solid line) and conventional loam (Δ dashed line). A) Survival of *E. coli* O157:H7 in soils amended with manure GMH. B) Survival of *E. coli* O157:H7 in soils amended with manure GML. C) Survival of *Salmonella* serovar Typhimurium 110 in soils amended with manure GMH. D) Survival of *Salmonella* serovar Typhimurium 110 in soils amended with manure SH. E) Survival of *Salmonella* serovar Typhimurium 119 in soils amended with manure GMH. F) Survival of *Salmonella* serovar Typhimurium 119 in soils amended with manure SH.

no difference except for the conventionally managed loam soil where rate of decline was higher when GMH was amended compared to when GML was amended (p=0.012). *E. coli* O157:H7 declined significantly faster (p<0.05) in all organically managed soils then in the conventionally managed neighboring soils, except for loam soil amended with GML. *E. coli* O157:H7 disappeared exceptionally rapid in the organic sandy soil (Fig. 4 and 5).

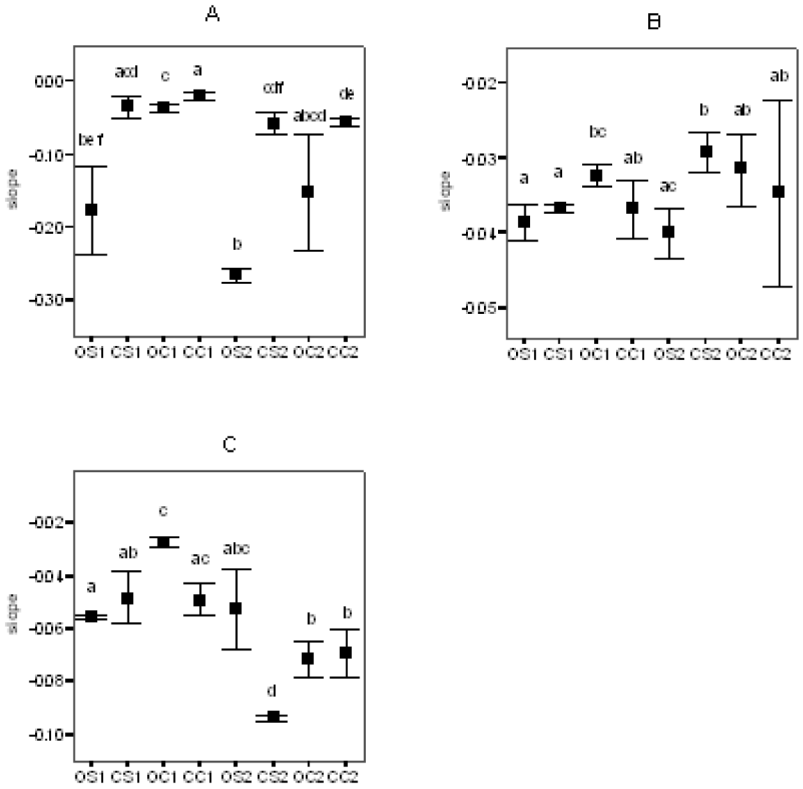

Fig. 5. Values of the estimated slope parameter for the survival of *E. coli* O157:H7 (A) and of *Salmonella* serovar Typhimurium MAE 110 (B) and 119 (C) in 4 different soils: organic sand (OS), conventional sand (CS), organic loam (OC) and conventional loam (CC). For *E. coli* O157:H7 these 4 soils were amended with manure type GMH (1) and GML (2) and fitted to an exponential decline model while for *Salmonella* they were amended with GMH (1) or SH (2) and fitted to a logistic decline model as with the survival in manure. Error bars show mean +/- 1.0 standard error of the mean. Treatments showing identical letters do not significantly differ.

The rate of decline in soils was positively correlated with total nitrogen content (r=0.86, p=0.006), nitrate content (r=0.81, p=0.014) and total carbon content (r=0.82, p=0.012). Stepwise multiple regression first resulted in a model, solely including the

total nitrogen content: slope [model R2=0.80] = -2.15 × 10-1 [total nitrogen; partial R2=0.80, p=0.105] + 2.29 × 10-1 [intercept]. When, excluding the total nitrogen content and parameters correlated with it (nitrate content and total carbon content), no variable was strong enough to enter the model en thus explaining a significant part of the variation in decline rate of *E. coli* O157:H7 in soil.

2.3.4 Survival of *Salmonella* serovar Typhimurium in soil

Densities of *Salmonella* serovar Typhimurium declined more steadily than *E. coli* O157: H7 and was in most cases still detected at 56 days after application of the manure to the soils (Fig. 4). Decline rates of *Salmonella* serovar Typhimurium could not be compared with *E. coli* O157:H7 directly because a different decline model was used. The two Typhimurium phenotypes showed a quite different pattern of decline rate over the treatments: the two phenotypes differed significantly from each other in decline rate in 5 of the 8 treatments ($p<0.05$) (Fig. 5). With *Salmonella* serovar Typhimurium phenotype 110 none of the manure-soil treatments was exceptional with respect to the decline rate. Phenotype 119 showed an exceptional fast decline in conventional sand amended with manure GH and a relative slow decline in organic loam with GMH, compared to the other treatments. No consistent differences were found between organic and conventional soils.

Rate of decline of phenotype 110 in soils amended with SH was positively correlated with nitrate content ($r=0.95$, $p=0.049$), total nitrogen content ($r=0.95$, $p=0.047$) and total carbon content ($r=0.99$, $p=0.007$). Rate of decline in soils amended with GMH did not show any correlations with soil characteristics.The rate of decline of phenotype 119 showed no correlations with any of the chemical parameters.Variation in the rate of decline of phenotype 110 over all treatments was best explained by a model solely including the total nitrogen content: slope [model R2=0.99] = -4.30 × 10-4 [total nitrogen; partial R2=0.99, p=0.071] -3.03 × 10-2 [intercept]. For phenotype 119 and when making no distinction between the phenotypes, no parameter entered the regression model.

2.3.4 Presence on or in lettuce

Only one root sample of a lettuce crop grown on conventional loam amended with manure type GMH showed presence of *E. coli* O157:H7 (1.5 log CFU gdw^{-1}). Because this sample was not surface sterilized it is not clear whether the pathogen was present in the rhizosphere, attached on the root surface or internalized in the root tissue. None of the samples were positive for *Salmonella* serovar Typhimurium phenotype 110 and 119.

2.4. Discussion

The potential presence of human pathogens like *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in vegetables, grown in soils enriched with manure, is of growing concern. We simulated the lettuce production chain in three consecutive steps, following the fate of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure, manure-amended soil and lettuce. This way the pathogens experience three different niches and two niche transitions which is a more realistic setup compared to focusing on survival in one particular niche or determining the association of pathogens with vegetables by planting them directly on inoculated manure-amended soil. We investigated the effect of different cattle diets, soil types and soil management types on pathogen survival in manure and soil.

We showed that roughage type, but not the dietary crude protein level, influences the survival capabilities both of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. Decline of *E. coli* O157:H7 was faster in manure derived from a pure straw diet (higher pH, higher fiber content) compared manure derived from to a high-digestible grass-silage / maize silage diet (lower pH, lower fiber content). Decline in manure derived from a pure low-digestible grass-silage was intermediate. Persistence of *Salmonella* serovar Typhimurium in manure was better than that of E. *coli* O157:H7. . Roughage type showed no effect on the rate of decline of *Salmonella* serovar Typhimurium, although decline was significantly faster in the manure derived from straw compared to the manure from the grass-silage + maize-silage diet. Decline rates of both pathogens were mainly determined by the pH and fiber content of the manure. After the first niche transition from manure to manure-amended soil both pathogens declined further and again *E. coli* O157:H7 declined faster compared to *Salmonella* serovar Typhimurium. *E. coli* O157:H7 declined exceptionally fast in the organically managed sandy soil. After survival in manure and manure-amended soil the final and most likely more realistic bacterial loads in the soils used in this experiment did not result in the presence of *E. coli* O157:H7 or *Salmonella* serovar Typhimurium in or on the edible parts of lettuce.

Survival times of *E. coli* O157:H7 reported in this study, ranging between 56 and 133 d at 10°C, resemble earlier published persistence times of *E. coli* O157:H7 in bovine manure (6, 26, 28). *Salmonella* serovar Typhimurium survived clearly longer than *E. coli* O157:H7 and was still present after 133 days. Theoretical elimination times of *Salmonella* serovar Typhimurium of 151 days at 4°C, 85 days at 20°C and 14 days at 37°C in bovine manure could be derived from linear regression equations (18). In general it is very difficult to compare survival studies due to the variety of experimental setups used. Moreover, like we showed with this study, survival times do not only depend on temperature but also on the manure composition which is determined by the feeding regime.

Cattle diet has been considered a potentially important factor in controlling presence of *E. coli* O157:H7 and *Salmonella* in cattle, given that it likely affects gut microbial populations (34) but results are not unambiguous. Considerable attention has been paid to the controversial effect of cattle diet on pathogen shedding by the animal (11, 20, 25, 38, 45, 48). Roughage type may not only be important in controlling shedding but also is important with respect to pathogen survival in manure. We showed that the human pathogens *E. coli* O157:H7 and *Salmonella* are more persistent in manure derived from cattle fed a diet characterized by a higher energy and lower fiber content (high-digestible grass-silage + maize-silage) compared to manure derived from a diet characterized by a lower energy and higher fiber content (straw). Feeding hay to cattle may be a way to reduce shedding of acid resistant *E. coli* (11). Diets high in grain are thought to create a more acidic rumen environment because the starch is incompletely digested and is fermented in the colon, which in turn should lead to the selection of more acid-tolerant *E. coli* (11, 38). It is known that both *E. coli* O157:H7 and *Salmonella* serovar Typhimurium possess several systems for surviving exposures to low pH and therefore can be considered to be quite acid-resistant (12, 7). Extrapolating to pathogenic *E. coli,* the results of Diez-Gonzalez (11) seems to be supported by some experimental studies (8, 45) and several epidemiological studies (16, 10, 37) which found a positive association between *E. coli* O157:H7 prevalence and the feeding of barley, corn silage and grains. Also *Salmonella* prevalence in dairy heifers was found to be lower when feeding hay (27). In contrast, some epidemiological studies (39, 47) and various studies using artificially inoculated animals seem to contradict the idea that more forage feeding (hay) compared to grain feeding is a mechanism to reduce selection for increased acid-resistance and *E. coli* O157:H7 shedding by ruminants (15, 20, 25, 48).

Although conditions in excreted manure are likely to be different compared to those encountered in the rumen environment, our results seem to agree with the proposition that a high energy diet containing grains/starch is favoring the proliferation and survival of *E. coli* O157:H7. We also showed the importance of a high fiber content of the diet and the resulting manure with respect to the elimination of human pathogens. This might be related to the combination of a relative slow release of readily available nutrients in manure with higher fiber content and the more copiotrophic nature of *E. coli* and *Salmonella*. In practice, feeding starch in the form of grains or maize is common practice in dairy farming in order to fulfill the energy need of high milk production. However, there is a trend in more sustainable and organic dairy farming of feeding a diet with increased fiber content consisting of lower concentrations of cytoplasmic carbohydrates (sugars, starch) and more so-called cell wall carbohydrates (hemicellulose, cellulose, lignin). This is often accompanied by a higher C/N ratio, consequently reducing nitrogen losses to the environment (49). According to our

findings this should result in lower survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium and consequently in a lower risk of transfer of these pathogens into the vegetable production chain.

The land application of infected manure is a major transition for human pathogens since soil can be considered to be a hostile environment for bacteria that have the gastro-intestinal tract of mammals as their primary habitat. Although pathogen levels gradually decline with increased storage time and after land application, it is recommended that an interval of at least 120 days (1) or even 6 months (31) should be observed between manure spreading and harvest of the crop. Our results of *E. coli* O157:H7 survival between 2 and 56 days in manure amended soil are comparable with earlier reported survival times of 34 days in sandy loam soil amended with cow manure at a similar temperature and almost similar manure to soil ratio (23). Others reported longer *E. coli* O157:H7 survival times between 154 and 217 days in soils amended with inoculated compost (21) and *Salmonella* serovar Typhimurium persistence between 203 and 231 days (22). However, these studies rely on inoculating the substrate with relatively high densities (> 10^5 CFU gdw^{-1}). In the present study we started monitoring the fate of the pathogens in manure-amended soil after they declined to relatively low and more realistic levels in manure (approximately 10^2 CFU gdw^{-1} for *E. coli* O157:H7 and 10^4 CFU gdw^{-1} for *Salmonella* serovar Typhimurium). As with survival in manure, it must be stressed that comparison between studies is difficult as different substrates and experimental setups are used. Persistence seems to depend on factors like temperature (23), manure-to-soil ratio (23), and soil type (32). We showed that decline of *E. coli* O157:H7 was faster in the organically managed soil compared to their conventional neighbor in 3 out of 4 cases and was exceptional fast in the organic sandy soil treatments. The latter may be more due to the relative high levels of nitrate, total nitrogen and total carbon in this specific organic sandy soil. This might have increased the activity of the native microbial population which decreased the competitive success of the introduced pathogen. The extremely fast decline in this particular soil was not observed for *Salmonella* serovar Typhimurium which may have a higher competitive ability. More research with more pairs of soils is needed in order to differentiate between organic and conventional soils with respect to human pathogen suppression.

The third transition, the planting of lettuce, did eventually not result in the presence of *E. coli* O157:H7 or *Salmonella* serovar Typhimurium on or in the edible parts of iceberg-lettuce. Some experimental studies demonstrated that these pathogens can become associated with vegetables (21, 22, 30, 54, 56). However, a wide variety of experimental setups was used (seedlings or seeds grown hydroponically or in soil) and most of these studies only proved surface contamination. Solomon (41) showed that *E. coli* O157:H7 can enter the lettuce plant from contaminated manure through the root

system and can migrate throughout the edible part of the plant. Recently, our laboratory also confirmed the possibility of internalization of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in iceberg-lettuce grown hydroponically and in inoculated soil (E. Franz , A.A. Visser, , A.D. van Diepeningen, M. M. Klerks, A. J. Termorshuizen and A.H.C. van Bruggen, submitted for publication). However, the numbers of bacteria used in these studies were far greater than what may be found in an agricultural field. In the current experiment the pathogen densities in the bulk soil at the time the lettuce was planted were approximately 10-100 CFU's gdw^{-1} for *E. coli* O157:H7 and 100-1000 CFU's gdw^{-1} for *Salmonella* serovar Typhimurium. These densities might be more realistic. Most likely, the population pressure was too low to allow the pathogens to enter the plants. Indeed, the results of Solomon (41) showed an increased number of positive samples with increasing pathogen density of the inoculum (10^4, 10^6 and 10^8 CFU's gdw^{-1}).

This study showed for the first time the fate of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium through subsequent niches: manure, manure-amended soil and manure-amended soil with lettuce. The results indicate that cattle feeding regime must be recognized as an important factor determining the survival of these pathogens in manure. Since manure is the primary fertilizer in organic vegetable production and is frequently used in conventional production, these results are of importance with respect to the microbial safety in vegetables production. Our results indicate that although manure is more frequently used in organic production, this does not automatically imply a higher risk of pathogen transfer to vegetable production. More work has to be done on how differences between organic and conventional farming may lead to differences in pathogen survival, not only in manure but in the whole farm ecosystem.

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Chapter 3

Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium

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Abstract

The primary objective of this study was to determine the possibility of internalization of GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium strains MAE 110 (multicellular morphology) and 119 (wild type morphology) into lettuce seedlings (*Lactuca sativa* cv. Tamburo) grown in an inoculated hydroponic and soil systems. Second aim was to quantify the level of contamination with the use of a proper surface sterilization method. Silver nitrate was superior in reducing the number of viable bacteria on leave surfaces compared to sodium hypochlorite + ethanol. With the hydroponic system internal colonization of lettuce only occurred at high densities with *S.* Typhimurium MAE 119. With the soil system *E. coli* O157:H7, *S.* Typhimurium 110 and *S.* Typhimurium 119 were found at considerable densities in sterilized leaf samples (respectively 3.95, 2.57 and 2.37 log CFU/g on average) with prevalences of respectively 0.29, 0.23 and 0.15. No statistical differences were observed between the *Salmonella* strains. A negative correlation was observed between shoot weight and leaf contamination. The observed presence of the used pathogens in lettuce, after thorough surface sterilization, demonstrates the possibility of the presence of human pathogens in locations were they are unlikely to be removed by consumer washing actions and therefore pose a serious threat when occurring in field situations.

3.1 Introduction

Vegetables and fruits are generally colonized by a wide variety of micro-organisms, such as bacteria, yeasts and fungi that cause spoilage (16). While human pathogenic bacteria were thought to be primarily associated with animal products, it is evermore recognized that human pathogens like *Shigella* spp., *Salmonella*, enterohemorrhagic *Escherichia coli*, *Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, and *Clostridium botulinum* may be associated with fresh produce (3). The documented disease cases show an increase in food-borne illness linked with fresh produce (24). *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) are typical examples of these human pathogens and outbreaks of both pathogens have been associated with the consumption of lettuce (7, 9).

Both *E. coli* O157:H7 and *S.* Typhimurium are considered to have a primary reservoir in cattle and are excreted in their manure (4, 25). Although conditions outside the animal host are considered to be unfavourable for growth, these pathogens may survive for extended periods of time in manure and manure-amended soils (8, 10, 11, 15). Human pathogens can be transmitted from contaminated soil to growing vegetables and can become surface associated with rucola and radish (17), and with lettuce and parsley (10, 11). The internalization of pathogens within growing vegetables which are eaten raw is of primary concern since those bacteria are protected against removal by washing. Internalization of human pathogens has been observed in various vegetables (12, 13, 21, 27, 28). With respect to lettuce, internalization of *E. coli* O157:H7 in the edible parts was shown in seedlings grown from seed in soil amended with contaminated manure, by selective plating following surface sterilization and by laser scanning and epifluorescence microscopy but no quantitative information was given with respect to the degree of contamination (21). A second study showed *E. coli* O157:H7 adherence patterns on lettuce seedlings by fluorescent and confocal laser scanning microscopy and quantitative selective plating (27). However, the greatest number of bacteria was associated with the roots and no differentiation could be made between internal and surface contamination based on plate counts because no thorough surface sterilization was applied. Recently Jablasone *et al.* (13) showed internalization of *E. coli* O157:H7 and *S.* Typhimurium by surface sterilization and quantitative plating. Positive samples were only found at one time point (9 d after inoculation) and in densities very close the detection limit. However, though some of these studies may suggest internalization of the human pathogens in plant parts, quantitative information on the prevalence and level of infection is scarce, if not absent.

Therefore, the objective of this study was to determine the possibility of internalization of human pathogenic bacteria in lettuce and to quantify the level of total contamination and internal contamination with the use of a proper surface sterilization. We used a GFP-expressing *E. coli* O157:H7 and two GFP-expressing strains of *S.* Typhimurium. One

strain of *S.* Typhimurium produces thin aggregating fimbriae resulting in a multicellular morphotype which plays a role in the attachment to surfaces, while the other strain displays the wild-type morphotype (18, 19). In addition we investigated potential difference in lettuce colonization between both strains of *S.* Typhimurium. We present the data in the form of probability distributions of the probability of infection and the probability of infection with a certain level of pathogen density.

3.2 Materials and methods

3.2.1 Bacteria.

E. coli O157:H7 B6-914 GFP-91 (further referred to as *E. coli* O157:H7) was used, which was constructed by Fratamico *et al.* (6) by transforming *E. coli* O157:H7 B6-914 with green fluorescent protein (GFP) plasmid pGFP (cDNA vector, Clontech). The strain does not produce Shiga-like toxins Stx1 and Stx2, is resistant to the antibiotic ampicillin and was chosen for this experiment because of laboratory safety advantages. The *eae* gene coding for intimin, which is involved in attachment, is still intact. No differences in growth kinetics between this strain and the parental strain have been detected (6). The lack of the toxin genes Stx1 and Stx2 did not influence the *E. coli* O157:H7 growth characteristics (15). Bacteria were stored at –80 °C, recovered and cultured on Sorbitol MacConkey Agar (Oxoid, Basingstoke, UK) supplemented with ampicillin (50 μg/ml) at 37°C. *E. coli* O157:H7 was subsequently grown in Luria-Bertani (LB) broth supplemented with ampicillin (50 μg/ml) at 37°C.

Two morphotypes of *Salmonella enterica* serovar Typhimurium, MAE 110 (Pagf D1, rdar: aggregate/multicellular phenotype) and MAE 119 (ΔagfD101, saw: wild type morphology), were kindly provided by Dr Ute Römling (18, 19). AgfD has been reported to regulate curli and cellulose production which are both required for multicellular behavior of *Salmonella* (18, 19). Both strains were derived from strains MAE52 and MAE 51 respectively and both contain kanamycin and gentamycin resistances and a GFP-gene on the chromosome after transformation with the PAG408 mini-transposon (22). The two strains can be distinguished by their appearance under UV-light: the colony shape of MAE 110 is larger, flatter, more ragged and less bright compared to MAE 119. Bacteria were stored at –80 °C, recovered and cultured on LB-medium supplemented with ampicillin (50 μg/ml) at 37°C. Both strains were subsequently grown in LB broth supplemented with ampicillin (50 μg/ml) at 37°C.

Bacteria were harvested from an overnight grown culture by centrifugation (9000 × *g* for 5 min at 20°C). Suspensions of 1×10^9 CFU/ml were made by resuspending the

pellet in 1% peptone buffer and diluting to an OD_{630} of 0.7 in the spectrophotometer which is equal to approximately 1×10^9 CFU/ml **.**

3.2.2 Effectiveness of surface-sterilization.

Lettuce (*Lactuca sativa* cv. Tamburo) seeds were sprouted in sterile Petri dishes with wet filter paper, at room temperature. Eight-days-old seedlings were used. Plants were divided in shoot and root by cutting the hypocotyls. Plant parts were dipped in GFP-*E. coli* O157:H7 solution containing 1×10^9 CFU/ml for 15 s and air-dried for 5 min. Plant parts were surface sterilized by 1) dipping in 1% $AgNO_3$ for 10 s followed by two washing steps of 10 s in demineralized water or by 2) dipping in 1% sodium hypochlorite (NaHClO) for 5 s followed by 5 s in 70% EtOH for 5 s and two washing steps of 10 s in demineralized water. Plant parts were then ground in a mortar with peptone buffer (.01%) and plated (100 µl) onto Soribitol MacConkey agar (SMAC) supplemented with ampicillin (50 µg/ml). Plants parts which were not surface sterilized were grounded and plated without dipping in sterilization solution and without washing in water. Plates were incubated overnight at 37°C and colonies were counted using a dark-blue lamp (Philips PL-S 9W/08 Blacklight Blue, peak at 365 nm UV-A). Colony shape and GFP-intensity enabled distinction between *S.* Typhimurium morphotypes 110 and 119.

3.2.3. Hydroponic experiment.

Seedlings were produced as described above**.** Eight-days-old seedlings were placed individually in 30 ml tubes containing 29 ml of 10% Hoagland nutrient solution (0.7 ml/L $Ca(NO_3)_2$ 1M, 0.50 ml/L KNO_3 1M, 0.20 ml/L KH_2PO_4 1M, 0.20 ml/L $MgSO_4$ 1M, 0.10 ml/L trace element solution, 0.10 ml/L FeEDTA). The roots of the seedlings were in the nutrient solution while the shoot was growing outside the tube. *E. coli* O157:H7, *S.* Typhimurium MAE 110 and 119 were added to the solution at a final density of 3.39×10^7 CFU/ml (0.5 ml inoculum). Each treatment consisted of 10 plants. Ten controls were made by adding 0.5 ml demineralized water to each control plant. Plants were incubated at room temperature. After 3, 7 and 10 d the solutions were stirred with a pipette to resuspend the remaining bacteria. After 10 d 2 ml of 10% Hoagland solution was added to each flask to compensate for evaporation. Plants were harvested 18 d after inoculation. The hypocotyl of each plant was cut just above the seal to separate the root from the leaf section. The leaf section was then separated in two halves. One half was surface sterilized with $AgNO_3$ (as described earlier), the other half was not surface sterilized. The roots were surface sterilized with $AgNO_3$. Homogenizing, surface sterilization, plating and counting were done as described earlier.

3.2.4 Soil experiment.

Seeds were placed 0.5 cm deep into watered potting soil. Pots were placed in a warehouse at 15°C and a relative humidity of 60%. The pots were watered three times a week and received crystal blue plant nutrition after 3 and 31 d. When more than one seed sprouted, one plant was retained and the other plants were carefully removed from the pot in such a way that all pots contained approximately equally sized seedlings. To 12 pots 3 ml 1×10^9 CFU/ml of *E. coli* O157:H7 or a mixture of *S.* Typhimurium MAE 110 and *S.* Typhimurium MAE 119 were added after 14 and 18 d, each time after watering and carefully avoiding surface contamination. To 12 pots no pathogens were added and served as controls. After 35 d the plants were harvested, the leaves were cut from the roots and total shoot weights were determined. Per plant two leaves which were not in direct contact with the soil were used; one was surface-sterilized with $AgNO_3$, the other was plated without surface-sterilization. Homogenizing, plating and counting were done as described earlier.

3.2.5 Statistical analysis.

For the "surface sterilization effectiveness test", the difference between the effectiveness of the two surface sterilization methods was examined by independent samples T-tests (SPSS Inc, Release 11.0.1). For the soil experiment non-parametric Mann-Whitney U tests were performed to detect differences between strains with respect to the number of infected plants and the level of infection (log CFU/g fresh weight). Correlation between contamination level and weight of the plants was done by a Pearson Correlation test. The relation between shoot weight and degree of contamination was described by fitting the data to a power model: $\log \text{CFU/g} = a \times \text{weight}^b$ (SAS Institute Inc, Cary, NC, USA).

Plant contamination can be seen as a binomial process with two possible outcomes: success (contaminated) or non-success (not contaminated). The binomial process consists of three parameters: the total number of trials n (in the soil system n=12), the number of infected plants s and the prevalence of infection p. Each parameter can be calculated when the other two are known. Typically, the prevalence (p) and the uncertainty around the true prevalence of shoot contamination can be assessed by the beta distribution: $p = \text{Beta}(s + 1; n - s + 1)$, where s is the number of infected plants within the treatment and n is the total number of plants in the treatment. A curve was produced by running a simulation in @Risk (version 4.5.4 Palisade Corporation) of 1000 iterations with Latin Hypercube sampling (Vose 2000). The result was plotted as a cumulative probability plot. This is very useful for reading off quantitative information about the distribution of p since one reads the probability of exceeding any value or

the probability of lying between two x-axis values, which is simply the difference between their cumulative probabilities. In contrast, a continuous probability density plot does not give one the actual probability of the corresponding x-axis value since that probability is zero but it represents the probability per x-axis unit (26).

The quantitative data on the shoot contamination (CFU/g) was log-transformed (only infected samples were included) and a cumulative frequency plot was produced as follows: the data was ranked in ascending order, for each value its cumulative percentile $P_x = i / (n + 1)$ was calculated where i is the rank of the data value and n is the total number of values and the data was plotted against the $i / (n + 1)$ values (*20*). A curve was produced by running a simulation in @Risk (version 4.5.4 Palisade Corporation) of 1000 iterations with Latin Hypercube sampling (26).

3.3 Results and discussion

Leaf surface sterilization with $AgNO_3$ showed significantly less remaining viable *E. coli* O157:H7 on the root and shoot surface than surface sterilization with sodium hypochlorite and EtOH ($p = 0.004$ for root and $p = 0.042$ for shoot). On average 25 CFU (log 1.40) and 204 CFU (log 2.31) per shoot remained after sterilization for respectively $AgNO_3$ and the combination of hypochlorite and EtOH. For root tissue these numbers were respectively log 1 and log 2.11 CFU/g. With the non-surface-sterilized shoots on average 3.6×10^6 CFU were detected per shoot. The leaf surface sterilization effectiveness was 99.9993% for the sterilization with $AgNO_3$ and 99.994% for the combination of hypochlorite and EtOH. Surface sterilization with $AgNO_3$ was considered to be superior (factor 10 better) and was used throughout this study.

With the soil system, the number of contaminated lettuce plants based on total pathogen presence was significantly higher for *E. coli* O157:H7 compared to *S.* Typhimurium 119 ($p = 0.024$). No differences were observed between *E. coli* O157:H7 and *S.* Typhimurium 110, and between *S.* Typhimurium 110 and *S.* Typhimurium 119 ($p>0.05$). The number of infected plants based on the data obtained after surface sterilization showed no differences between the strains ($p > 0.05$). The prevalence of colonized lettuce shoots based on the non-surface-sterilized samples was higher (for all three pathogens) than the prevalence based on the surface sterilized shoot samples (figure 2). *E. coli* O157:H7 showed the highest contamination prevalence for the non-sterilized shoots and the sterilized shoots ($p=0.93\pm0.07$ and $p=0.29\pm0.12$ respectively), followed by *S.* Typhimurium 110 ($p=0.69\pm0.12$ and $p=0.23\pm0.11$ respectively) and *S.* Typhimurium 119 ($p=0.54\pm0.13$ and $p=0.15\pm0.09$ respectively). The difference between the prevalence based on the shoot assay with surface sterilization (showing pathogens residing internally or in protected subsurface locations) and without

(showing the total presence of pathogens) indicates the prevalence of plants where the pathogens were only associated with unprotected surface locations. This was also highest for *E. coli* O157:H7 (0.64) followed by *S.* Typhimurium 110 (0.46) and *S.* Typhimurium 119 (0.39).

When infected, the degree of total contamination was highest for *S.* Typhimurium 119 (mean = 3.90±1.49log CFU/g), followed by *S.* Typhimurium 110 (mean = log 3.68±1.78 log CFU/g) and *E. coli* O157:H7 (mean = log 3.17±1.29 log CFU/g) (figure 2). The contamination levels of *S.* Typhimurium 119 and *E. coli* O157:H7 almost differed significantly (p=0.050). The difference between the Typhimurium morphotypes and the difference between *S.* Typhimurium 110 and *E. coli* O157:H7 were not significant (p = 0.767 and p = 0.146 respectively). In contrast, the degree of internal or subsurface contamination (as measured after surface sterilization) was highest for *E. coli* O157:H7 (mean = 3.95 ± 1.02 log CFU/g, s.d.=1.02), followed by *S.* Typhimurium 110 (mean = 2.57 ± 0.27 log CFU/g) and *S.* Typhimurium 119 (mean = 2.37 ± 0.30 log CFU/g). Significant differences were observed in the level of internal contamination between *S.* Typhimurium 110 and *E. coli* O157:H7 (p = 0.004), and between *S.* Typhimurium 119 and *E. coli* O157:H7 (p = 0.001). The Typhimurium morphotypes did not differ significantly (p = 0.387). For *E. coli* O157:H7 we found a higher average degree of contamination when leaves were surface-sterilized compared to non-surface sterilized leaves. This was unexpected since the CFU counts of the non-surface-sterilized samples include bacteria on the surface and inside the leaf tissue. The CFU counts of surface-sterilized samples should only encompass internalized bacteria. Since we sampled different leaves of one infected plant for plating after surface-sterilization and after no sterilization, our results indicate that with some colonized plants some leaves were more contaminated internally relative to externally. There is a possibility that we underestimated the amount of CFU due to the fact that direct plating on SMAC after surface sterilization is not accounting for the recovery of injured cells and because the use of peptone has been shown to result in a lower recovery of stressed cells (14). Future research should focus on the recovery of stressed and injured cells from lettuce tissue.

No microscopy was used to determine the exact location of the pathogens detected after thorough surface sterilization, but the fact that these bacteria were still present at relatively high densities after applying thorough surface sterilization (AgNO3 killing of rate of 99.9993%) means that they were located in protected subsurface or internal locations. When such colonization occurs under natural conditions, the presence of these bacteria at these locations pose a severe risk to the consumer since it is very unlikely that these bacteria are removed by consumer washing actions prior to consumption. In addition, it has been shown by confocal scanning laser microscopy that *E. coli* O15:H7 and *Salmonella* Typhimurium penetrated lettuce leaves to an average of 101 μm below the surface (23) and 60-80 μm below the surface of carrot tissue (1).

Although several studies showed association of human pathogens with lettuce or related vegetables (10, 11, 13, 17, 21, 27), only few gave indications of internalization (13,21,27).An effective sterilization method is needed to prove internal contamination. Solomon et al. (21) sterilized (5 s in 80% ethanol, 5 or 10 min. in 0.1% HgCL2 followed by washing with sterile water) the leaf surface of seedlings, grown from seeds in contaminated (104, 106 and 108 CFU/g) mixtures of soil and inoculated manure. Seedlings were either placed directly on selective medium or were sliced longitudinally and the inner surfaces were placed on plates. Results suggested that *E. coli* O157:H7 was located within the seedling tissue and therefore was protected against the sanitizing agent. Laser scanning confocal microscopy showed the pathogen in high numbers at subsurface locations, down to 45 μm below the tissue surface. However, quantitative information on the degree of contamination was not gathered. Jablasone et al. (13) surface sterilized (20 min. sodium hypochlorite) lettuce leaves of seedlings, grown in solidified hydroponic nutrient solution from inoculated seeds (102 CFU/ml) and plated the macerated samples on selective media. *E. coli* O157:H7 and S. Typhimurium were shown to be internalized, but only 9 d after inoculation and at low densities (resp. 1.47 ± 0.2 and 1.39 ± 0.4 log CFU/g), which are close to the detection limit of the method used. Surface contamination 9 d after inoculation was found to be around 5.92 ± 0.08 log CFU/g for *E. coli* O157:H7 and 5.59 ± 0.05 log CFU/g for S. Typhimurium. Finally, Wachtel et al. (2002) studied the association of *E. coli* O157:H7 with lettuce seedlings in a hydroponic system (seedling inoculation with approx. 1*106 CFU/ml, overnight exposure) and in a soil system (seed inoculation with a range of densities, 10 d of exposure). Seedlings were homogenized without thorough surface sterilization and plated on selective medium. The pathogen was found to be primarily associated with roots and seed coats in both systems, although some association was found with cotyledons close to the petiole and in close proximity to a stomatal pore. The authors state that fluorescent enterohemorrhagic *E. coli* was observed moving within the vascular system of the hypocotyls but this data was not shown. With the present study, using a thorough surface sterilization and quantitative methods, we showed the presence of *E. coli* O157:H7 and S.Typhimurium in internal or protected subsurface locations of lettuce plants and with a considerably high prevalence. This high prevalence of presence in internal or protected subsurface locations in combination with the relatively high amount of cells indicates a serious health threat linked to the consumption of fresh produce.

For each pathogen it was observed that those plants which showed internal contamination weighed less than plants without internal contamination. No significant differences in shoot weights between the pathosystems were observed. Over all treatments, a negative correlation was observed between shoot weight and leaf contamination

(non surface-sterilized leaves: r = -0.56, p = 0.001; surface-sterilized leaves: r = -0.52, p = 0.002). The negative relation between shoot weight and contamination was best fitted with a power model (log CFU/g = a × weightb) for both non surface-sterilized (p = 0.000, R2 = 0.64, a = 3.1281, b = -0.2924) and surface-sterilized leaves (p = 0.024, R2 = 0.60, a = 0.8527, b = -0.7650) (figure 1). The negative correlation between plant weight and the level of contamination of lettuce grown in soil indicates a plant-microbe interaction leading to reduced plant growth when colonized with human pathogens, which is a strong indication of the internal presence of the bacteria detected after surface sterilization. In this case, we might even speak of infection instead of contamination or colonization. No such effects on plant growth would be expected when the bacteria were only localized on the surface of the plant.

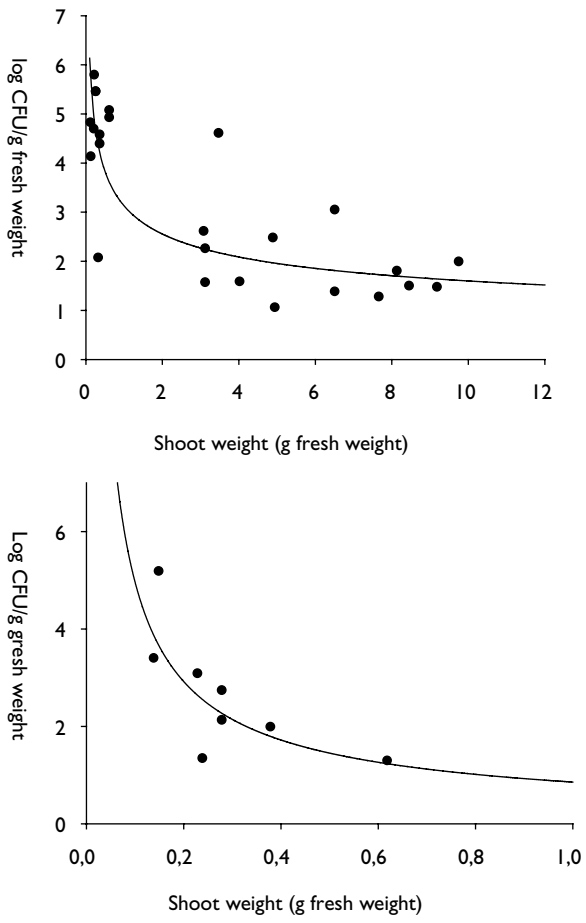

Fig. 1. Observed values (symbols) and fitted power function (line) representing the relation between contamination (*E. coli* O157:H7, S. Typhimurium MAE 110 and S. Typhimurium MAE 119 together; CFU/g fresh weight tissue) and shoot dry weight measured in non-surface-sterilized leaves (A) and surface-sterilized leaves (B).

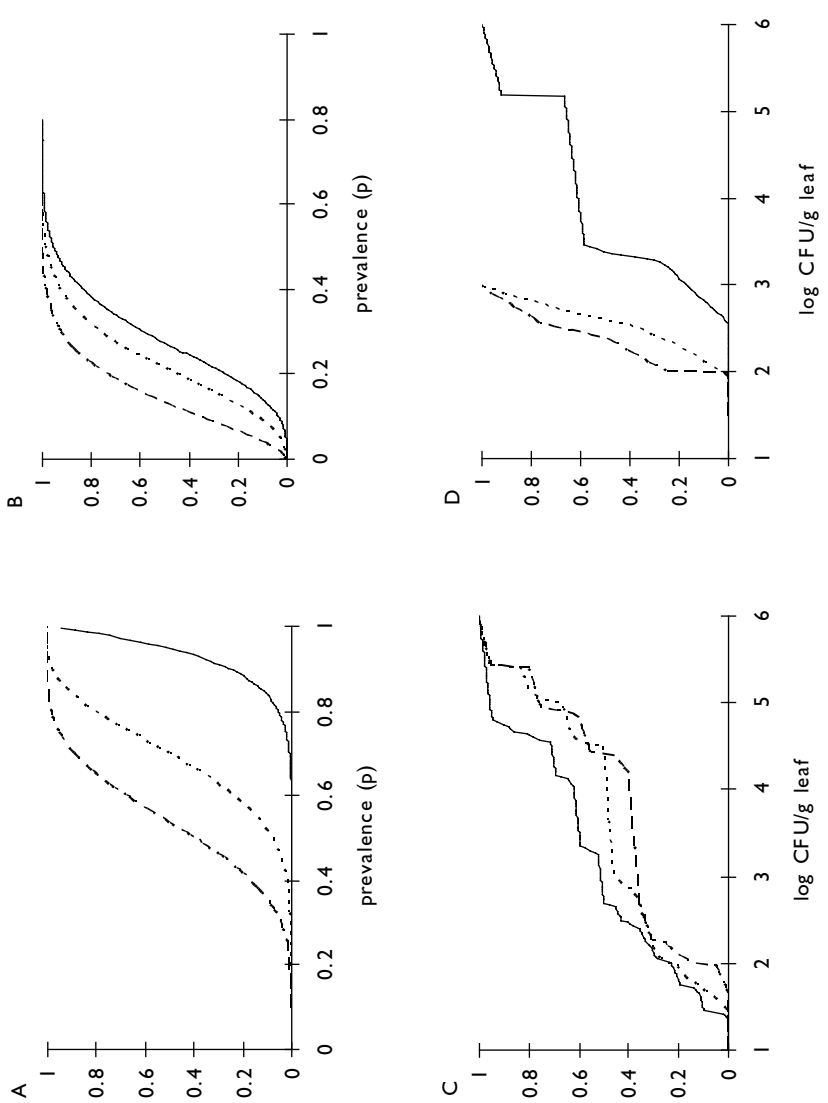

Fig. 2. Cumulative probability of prevalence (p) of lettuce shoot contamination by *E. coli* O157:H7 (solid lines), S. Typhimurium MAE 119 (wild type morphology, striped lines) and S. Typhimurium MAE 110 (multicellular morphotype, dotted lines), when grown in contaminated potting soil, followed by not applying (A) and applying (B) surface sterilization. The cumulative probability of the degree of contamination is shown after not applying (C) and after applying surface sterilization (D) (CFU/g dry leaf tissue). The graph should be read as the probability of exceeding a x-axis value or the probability of lying between two x-axis values. The latter is the difference between their cumulative probabilities.

With the hydroponic system, *S.* Typhimurium MAE 119 was detected in non-sterilized leaf, sterilized leaf and sterilized root samples in approximately equal numbers (Table 1). *S.* Typhimurium MAE 110 was detected in non-sterilized leaf and sterilized root samples but not in sterilized leaf samples. *E. coli* O157:H7 was not detected in any of the samples. The growth circumstances of lettuce apparently have an effect on the occurrence the contamination by the pathogenic bacteria. While *E. coli* O157:H7 did not infect the inside of lettuce in the hydroponic experiment, it was abundantly present in lettuce plants grown in potting soil. It could be that *E. coli* O157:H7 is more dependent on root damage (which is more likely to occur when plants are grown in soil than when grown hydroponically) in order to enter the plant, compared to the *S.* Typhimurium.

Table I. Quantitative and qualitative internal and external contamination of ice-berg lettuce parts/g[1] dry weight tissue grown for 18 d in a hydroponic system containing 3.39×10^7 CFU/ml of the respective pathogens.

Pathogen	Plant part	# positive plants / total	CFU count/g
E. coli O157:H7	leaf non-sterile[1]	0/10	0
	leaf sterile[2]	0/10	0
	root sterile	0/10	0
S. Typhimurium MAE110	leaf non-sterile	1/10	6.09×10^0
	leaf sterile	0/10	0
	root sterile	3/10	$5.57 \pm 1.06 \times 10^2$
S. Typhimurium MAE119	leaf non-sterile	4/10	$1.23 \pm 2.57 \times 10^5$
	leaf sterile	2/10	$1.22 \pm 2.57 \times 10^5$
	root sterile	4/10	$4.61 \pm 9.76 \times 10^5$

[1] Non-sterile: grounded and plated directly.

[2] Sterile: surface-sterilized with $AgNO_3$ for 10 s followed by two times washing for 10 s in demineralized water.

The two *S.* Typhimurium strains used in this study differed in their (colony) morphology. Strains 110 (AgfD) shows multicellular behavior by the production of aggregative fimbriae while strain 119 (lacking AgfD) shows the so-called wild type behavior. Recently, it was suggested that these fimbriae play an important role in the attachment and colonization of plant tissue (2). In this study the strain lacking the production of fimbriae was more successful in colonizing lettuce in a hydroponic system while in the soil system no differences in colonization capacity were observed between the strains. Comparison of the studies is difficult since totally different experimental setups were used and clearly more research is needed in this area to determine bacterial characteristics that have an important role in plant colonization.

Raw vegetables are increasingly recognized as a potential vehicle for human pathogens (3, 20, 24). Especially the presence of human pathogens at locations where they are protected from washing actions is of concern. This is of particular importance in Europe since no post-harvest chemical decontamination of vegetables like lettuce is allowed. The growth of vegetables in soils enriched with contaminated manure is thought to be the primary contamination mechanism. With the use of a proper surface sterilization method we showed that the human pathogens *E. coli* O157:H7 and *S.* Typhimurium can indeed become present at considerably high levels at internal or subsurface locations were they are protected against sterilization. These bacteria possess a serious health threat to the consumer since it is very unlikely that these bacteria are removed by consumer washing handlings. Moreover, in Europe no disinfection of vegetables in allowed. Although these experiments showed convincingly that *E. coli* O157:H7 and *S.* Typhimurium are capable of infecting lettuce plants in high densities, the study presented here is still a worst-case scenario with extreme exposure to pathogens. There are indications that contamination is not so prevalent at lower, more realistic pathogen densities in soil (5). Future work should focus on the possibility of contamination under more realistic conditions as found in the production fields.

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Chapter 4

Prevalence of Shiga toxin-producing *Escherichia coli stx1, stx2, eaeA* and *rfbE* genes and survival of *E. coli* O157:H7 in manure from organic and low-input conventional dairy farms

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Abstract

Manure samples were collected from 16 organic (ORG) and 9 low-input conventional (LIC) Dutch dairy farms during August and September 2004 to determine the prevalence of the STEC virulence genes *stx1* (Shiga-toxin 1), *stx2* (Shiga-toxin 2) and *eaeA* (intimin) and the *rfbE* gene which is specific for *E. coli* O157. The *rfbE* gene was present at 52% of the farms. Prevalence of *rfbE* was higher at ORG farms (61%) compared to LIC farms (36%) but this was not significant. Relatively more LIC farms were positive for all STEC virulence genes *eaeA*, *stx1* and *stx2*, which form a potentially highly virulent combination. *Enterobacteriaceae* species richness, determined by DGGE, was significantly lower in manure positive for *rfbE*. Survival of a green fluorescent protein-expressing *E. coli* O157:H7 was studied in the manure from all sampled farms and was modelled by a biphasic decline model. The time needed to reach the detection limit was predominantly determined by the level of coliforms and the pH (both negative relations). Initial decline was faster for ORG manure but leveled off earlier, resulting in a longer survival compared to LIC manure. Although the nonlinear decline curve could theoretically be explained as the cumulative distribution of an underlying distribution of decline kinetics, it is proposed that the observed nonlinear biphasic pattern of the survival curve is the result of a changing nutrient status of the manure over time (and thereby a changing competition pressure), instead of the presence of subpopulations differing in the level of resistance.

4. 1 Introduction

Shiga-toxin producing *E. coli* (STEC) is an important group of zoonotic human pathogens with *E. coli* O157 being the best known and most studied serotype (11). STEC are generally carried asymptomatically by cattle and shed in their faeces, which in turn may serve as a means of maintenance and spread of these pathogens among cattle herds (49). Since the reported increase in food-borne disease associated with the consumption of fresh vegetables (53, 56), the potential contamination of vegetable crops that are grown in fields enriched with manure has raised increasing concern (19, 30, 54). Understanding on-farm survival and spread of human pathogens is of utmost importance in preventing the spread of this organism to the environment, groundwater, food, crops and back to cattle.

A considerable body of literature exists on the prevalence of STEC on cattle farms and statistically associated management factors which could be used for potential intervention strategies to reduce shedding of these pathogens (3, 55, 60). However, these studies are not unambiguous and have resulted in the identification of only very few risk factors, like season and cattle age, which are ubiquitous. The direction of influence of the majority of the management practices like feeding regimen and housing conditions remain unclear and are under debate.

Dairy farm management practices (i.e. diet, housing conditions, antibiotic use) influence both the abiotic and biotic characteristics of the digestive system of cattle, the manure produced by these animals and the environment in which the pathogens are excreted. In turn, these abiotic and biotic factors will determine the susceptibility of cattle to STEC colonization, growth and/or survival in the ruminant gut and subsequent survival in excreted manure. To date little is known about the relation between STEC prevalence in manure and the chemical and biological composition of this complex substrate.

E. coli O157:H7 is able to survive for extended periods of time in manure but very little is known about the factors determining the fate of the pathogen in manure (20, 34, 62). Behaviour of pathogens in broth culture as function of different chemical and physical variables can not be extrapolated to complex substrates like manure. Recently it was reported that the pH and fibre content of the manure were the main determinants of survival of *E. coli* O157:H7 in manure derived from cattle fed different diets, with longest survival at lower pH and lower fibre content (20). However, there is still the need of studying pathogen survival under a wider array of non-experimentally produced manure in order to identify the driving factors behind pathogen survival and decline in manure.

Besides the chemical composition there are strong indications that the microbial community has an important influence on the survival capabilities of *E. coli* O157:

H7 in environmental substrates like manure. *E. coli* O157:H7 growth kinetics were influenced by presence of a competitive microflora compared to pure culture (15) and *E. coli* O157:H7 survived significantly longer in manure-amended autoclaved soil than in manure-amended non-autoclaved soil (31). In addition, the decline rate of the soil-borne plant pathogenic bacteria *Ralstonia solanacearum* in soil was positively correlated with the microbial diversity as estimated by DGGE (39). However, the influence of the microbial community on the survival of *E. coli* O157:H7 manure has never been investigated.

In order to conduct risk analysis on pathogen spread in the environment and the (vegetable) food chain, models are needed which describe accurately pathogen survival in complex substrates of concern. Many bacterial inactivation curves do not seem to obey first-order kinetics but show initial shoulders and tails or upward or downward concavity. Survival of human pathogens in manure is also often nonlinear, but attempts to model it accordingly are scarce (4, 20).

The main objectives of this study were (i) to determine the natural prevalence of the *E. coli* O157 specific *rfbE* gene and STEC virulence genes *stx1* (Shiga-toxin 1), *stx2* (Shiga-toxin 2) and *eaeA* (intimin) in manure from 16 organic and 9 low-input conventional dairy farms, (ii) to study the survival of an introduced *E. coli* O157:H7 strain in these manures and (iii) to relate the prevalence of the STEC genes and the survival characteristics to chemical and biological characteristics of the manure.

4.2 Materials and methods

4.2.1 Farm sampling

During August and September 2004 manure samples were collected from 9 low-input conventional (LIC) dairy farms throughout the Netherlands and 16 organic dairy (ORG) farms in the province Friesland of the Netherlands. All the ORG farms were certified organic and were joined in the Bioveem project which aims at the development of solutions for organic dairy farming. The LIC farms were joined in the VEL & VANLA (VV) project which is a joint initiative of two environmental corporations with the aim to enhance the knowledge concerning nutrient and manure management in order to develop solutions for sustainable dairy farming. Farms within the VV project may not be considered as typical conventional dairy farmers; farmers within this project were chosen because of the willingness to participate and because of the existence of a database of management practices. From a herd of lactating cows at each farm a number of faecal samples (depending on the herd size) were collected from the stable floor in plastic pots and were pooled according to the sampling protocol used

by Bouwknegt *et al*. (8). None of the farms used antibiotics at the time of sampling. Pooled samples were transported to the laboratory in closed plastic pots, stored at 4°C and microbiological analyses were started within 48 h after sampling.

4.2.2 Detection methods

Each pooled sample was enriched by adding a portion of 20 g manure from each pooled sample to 180 ml modified EC broth containing novobiocin (0.02 g L-1) (Fluka Chemie GmbH, Switzerland). Enrichments were incubated overnight at 37°C on a rotary shaker (100 rpm). Total DNA was extracted from 300 µl of the enrichment culture with the Bio101 FastDNA1 SPIN Kit for soil according to the manufacturer's specifications (Bio101, Carlsbad, CA, USA), except that bead beating (three times 90 s with four 1mm glass beads) was used instead of the FastPrep1 device.
For the detection of the *E. coli* O157 specific *rfbE* gene and STEC virulence genes *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2) and *eae* (initmin), the DNA samples were subjected to two separate Taqman PCR assays. The first assay allowed the simultaneous detection of *stx1*, *stx2 eaeA*. Primers for this assay were derived from Ibekwe et al. (29) and Sharma et al. (52). The second assay allowed the detection of the *rfbE*-gene, which codes for the lipopolysaccharide O side chain of *E. coli* O157 (18). To reduce false negative results, an internal amplification control (IAC) was used in the PCR assays mentioned above (33). Detection of this IAC was based on the co-amplification of a green fluorescent protein-gene (GFP-gene) from the genetically modified *Escherichia coli* strain 99507GFP. Sub-optimal amplification due to inhibitors is identified by a shift of the IAC Ct-value from Ct 31.5 (optimal) to Ct-values up to 40. PCR conditions were identical to the ones described in Klerks *et al*. (33). For each sample the increase in fluorescence was measured during amplification with an ABI 7700 sequence detector (Applied Biosystems, Foster City, USA). Thresholds (the minimal fluorescence above which a sample is determined positive) were calculated for both the target and the IAC by taking the average of the fluorescence of the negative controls plus four times the standard deviation of the fluorescence of these negative controls. For a positive sample this gives a 99% probability of being positive.

4.2.3 Manure characterisation

Coliforms.

Two subsamples of approximately 1.5 g of fresh manure from each pooled sample were collected in tubes containing 4.5 ml 0.1% peptone buffer. These bacterial suspensions were mixed by vortexing, sonicated in an ultrasonic cleaner (Bransonic 12, Branson Cleaning Equipment Co., Shelton, CT) for 30 s and again vortexed. Samples were taken

for further 10-fold serial dilutions. From two appropriate dilutions 50 µl was plated in triplicate on MacConkey agar (Oxoid CM0007). Total number faecal coliforms (facultative anaerobic gram negative, rod-shaped, non spore forming bacteria present in the intestinal tract of warm-blooded animals that can ferment lactose to gas and acid within 48 h at 35°C) were counted after 24 h at 37°C.

Lactic acid bacteria.

An estimation of the total number of lactic acid bacteria was made by taking samples as described for coliforms and plating them on MRS agar (Oxoid CM0361) using the double layer technique as indicated by the manufacturer. Numbers of CFUs were counted after incubation for 2 days at 35°C.

Chemical characterization.

Dried samples (40°C) were ground and analyzed for total carbon by the Dumas method followed by detection by a Fisons Type EA 1108 Element Analyzer (Milano, Italy) and for fibre content (59). Total dissolved nitrogen content (N_{ts}) was measured in a segmented-flow analysis system (Skalar analytical BV, the Netherlands). The dissolved organic nitrogen content (DON) was calculated by subtracting the amount of nitrogen present in ammonium and nitrate from the total dissolved nitrogen content. Nitrogen content was determined by the Kjeldahl method (10) and ammonium content was determined in a solution of trichloro-acetic-acid (TCA) by an Autoanalyzer II (Technicon Instrument Corporation, Tarreytown, NY). The pH was measured in a watery suspension with an Inlab pH level 1 (WTW GmbH, Weilheim, Germany).

4.2.4 DGGE.

DNA was extracted from 300 mg (fresh weight) manure sample with the Bio101® Systems FastDNA® SPIN® Kit for Soil according to the manufacturer's specifications (Qbiogene, Inc., Carlsbad, CA, USA) except that bead beating (three times 90 s) was used instead of the FastPrep1 instrument. The 16S rRNA gene of eubacteria and Enterobacteriaceae were amplified from manure DNA with the eubacterial primer pair U968-GC and L1401 (17) and the Enterobacteriaceae primer pair DG74f and RW01r (24) to which a GC-clamp according to (17) was added. The eubacterial PCR was performed using a touchdown scheme (46) for 30 thermal cycles and the enterobacterial PCR was performed using 3 min of denaturation at 95°C, whereafter 30 thermal cycles of 1 min at 95°C (denaturation), 1 min at 55°C (annealing) and 2 min at 72°C (extension) were performed and finished by an extension step at 72°C for 10 min. The PCR products were examined by standard 1.2 % (w/v) agarose-0.5x Tris-borate-EDTA (TBE) gel electrophoresis with ethidium bromide staining, to confirm

product integrity and size. DGGE was performed using the DCode system (Bio-Rad Laboratories, Hercules, CA, USA). We used 6% acrylamide gels (37.5 acrylamide:1 bisacrylamide) with a 45–60% denaturing gradient as defined by Muyzer et al. (40) to separate the generated amplicons (100% denaturant is 7 M urea and 40% formamide) and an 8% acrylamide stack without denaturing agents. The gels were poured from the top in the DCode template, prepared with Gelbond PAG film (Amersham Pharmacia Biotech AG, Uppsala, Sweden) to one side, using a gradient maker and a Heidolph Pumpdrive (Heidolph, Schwabach, Germany) set at 4 ml/min. Eubacterial and enterobacterial PCR products derived from DNA of each manure sample were loaded in adjacent slots.

Electrophoresis was performed in 0.5x TAE buffer for 16 h at 100 V at a constant temperature of 60°C. Gels were stained with Bio-Rad's Silver Stain (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol, but using the protocol for gels >1 mm thick instead of 0.5–1 mm to compensate for the barrier formed by the Gelbond. After staining the gels were preserved for at least 1 h in Cairn's preservation solution of 25% ethanol (v/v) and 10% glycerol (v/v), covered by a permeable cellophane sheet (Amersham Pharmacia Biotech Ag, Uppsala, Sweden) and dried overnight at 60°C.

The gels were scanned using ScanSoft Omnipage pro. 14 at a resolution of 300 dots per inch. Scanned gels were analysed with Phoretix 1D (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). Bands were selected manually. Data of different DGGE gels were standardized by referring to the DGGE marker. The bacterial diversity was estimated in two ways: as species richness *S*, and as the Shannon-Wiener index of bacterial diversity, *H*. Species richness *S* was defined as the number of DGGE detected bands per soil type (58). The Shannon-Wiener diversity index was calculated as:

$$H = -\sum_{i=1}^{S} p_i \log p_i = -\sum_{i=1}^{S} (N_i / N) \log (N_i / N)$$

where P_i is the importance probability of the bands in a gel lane, S is the individual band, N_i is the band intensity for each individual band and N is the sum of intensities of bands in a lane. DGGE analysis was done in duplicate where the replicas were on different gels. S and H were calculated as the mean of the two replicas.

4.2.5 Survival experiment

Strain.

Strain *Escherichia coli* O157:H7 B6-914 GFP-91 was kindly provided by Dr. Pina Fratamico (Wyndmoor, USA). This strain does not produce the Shiga-like toxins I and

II ($Stx1^-$ $Stx2^-$), and contains the pGFP cDNA vector (Clontech Laboratories, Inc. Palo Alto, CA) expressing green fluorescent protein (GFP) and ampicillin resistance. The survival characteristics of this strain were indistinguishable from those of the wild-type strain (21). Kudva *et al.* (34) reported no differences in survival in bovine manure and manure slurry between toxin positive ($Stx1^+$ $Stx2^+$) and toxin negative ($Stx1^-$ $Stx2^-$) *E. coli* O157:H7. In addition, survival in manure of *E. coli* O157:H7 that passed the intestinal tract of cattle was not different from the survival of the same strain directly inoculated into the manure (50). Bacteria were stored at –80°C and checked for viability prior to use by growing on Luria-Bertani medium supplemented with ampicillin (50 μg ml^{-1}).

Inoculation and sampling of manure.

The pooled samples were mixed per farm and the survival experiment was executed with two replicas per farm. Preparation of inoculum, inoculation of manure and sampling over time were done as described in detail by Franz *et al.* (20). In brief, cells suspended in buffered peptone water (BPW) were added to 200 g manure with a final density of 1×10^7 CFU per gram manure dry weight (gdw^{-1}), taking the individual dry weights of the different manures into account. The manure and the inoculum were thoroughly mixed by kneading in a plastic bag from the outside by hand. Subsequently, the inoculated manure was transferred to plastic pots (1 L) which were closed (but with the ability of gas exchange) and incubated at 10°C in darkness. Moisture content remained constant (85%±3) during the experiment. The inoculated pots were sampled over time using serial dilution series with BPW, sonication in a ultrasonic bath for 30 s (Branson 5200, 120W output power, 47 kHz) and plating on Sorbitol MacConkey (SMAC, Oxoid CM813) agar supplemented with ampicillin (50 μg ml^{-1}). Numbers of fluorescent CFUs were determined using a dark-blue lamp (Philips PL-S 9W/08 Blacklight Blue, peak at 365 nm UV-A) after incubation for 18-20 h at 37°C.

4.2.6 Statistical analysis

Prevalence.

Farm level prevalence of *E. coli* O157 and STEC virulence genes were calculated as the ratio between the number of farms showing at least one positive pooled sample and the total number of farms. Chi-square analysis was used to test for differences in proportions of positive farms between ORG and LIC manure (PROC FREQ, SAS® system for Windows version 8.02, SAS Institute Inc, Cary, NC, USA, 2001). Based on the number of positive farms the true prevalence p with associated uncertainty was modelled with a beta-distribution: $p = (s+1) / (n-s+1)$, where s is number of positives and n the total number of farms or samples. True prevalence and 90% confidence

interval were running 1000 iterations with Latin Hypercube sampling (61) with @ Risk software (version 4.5.4 Palisade Corporation, Newfield, NY, USA).

Fitting of survival data.

Plate counts of zero were replaced by 10 CFUs gdw^{-1}, which is the calculated detection limit of the dilution plating procedure. The microbial survival data of both replicas of each farm were averaged. The majority of the survival curves clearly showed shoulders, tails and a biphasic pattern. Clearly, first-order kinetics were not appropriate. Therefore, the log-transformed survival data was fitted to a biphasic model as proposed by Geeraerd *et al.* (22) with the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT):

$$\log CFU(t) = \log(N_0) + \log\left(\begin{array}{l}(f \cdot e^{-k\max 1 \cdot t} \cdot \dfrac{e^{k\max 1 \cdot S1}}{1 + (e^{k\max 1 \cdot S1} - 1) \cdot e^{-k\max 1 \cdot t}} + \\ (1-f) \cdot e^{-k\max 2 \cdot t}) \cdot \left(\dfrac{e^{k\max 1 \cdot S1}}{1 + (e^{k\max 1 \cdot S1} - 1) \cdot e^{-k\max 1 \cdot t}}\right)^{\dfrac{k\max 2}{k\max 1}}\end{array}\right)$$

where t is time in days, N_0 is the number of cells present at t0, f is the fraction of the initial population in a major less resistant subpopulation, (1-f) is the fraction of the initial population in a minor more resistant subpopulation (at t0), k_{max1} and k_{max2} (day^{-1}) are the specific inactivation rates of the two subpopulations and sl is the initial shoulder length (time). In addition to the evaluation of the separate model parameters, the time necessary to reach the detection limit (*ttd*) was calculated with the biphasic model and subjected to the same statistical analysis as described for the individual parameters. Model performance was evaluated by assessing the Root Mean Square Error

$$(RMSE = \sqrt{((\sum (X_{sim} - X_{obs})^2)/N})$$

and the pseudo-R^2 regression coefficient

$$(R^2 = 1 - SS_{residuals} / SS_{total[corrected]}).$$

The standard error of estimated parameters and residuals were assessed visually for each fit and were checked for large values.

Data analysis survival

ORG versus LIC manure and PCR-positive versus PCR negative manure were compared with respect to the estimated parameter values of the survival models and

the chemical and biological variables by nonparametric Mann-Whitney tests since very few of the parameters and variables were normally distributed (SPSS v 12, SPSS Inc., Chicago, Illinois, USA). Nonparametric correlation tests (Spearman) were conducted to test for linear relations between model parameters and measured variables (SPSS). Regression analysis was conducted in order to describe possible nonlinear relations (SPSS). Multiple regression models that describe the expected values of the model parameters as function of biotic and abiotic manure characteristics were constructed (stepwise method, significance level p=0.15) (SAS® system for Windows version 8.02, SAS Institute Inc, Cary, NC, USA, 2001). The following variables were considered: pH, dry matter content in percentage of total weight (DM), nitrate content (NO_3), ammonium content (NH_4), total nitrogen content (N_{total}), total dissolved organic nitrogen content (DON), total dissolved organic carbon content (DOC), acid detergent fibre content (ADF), neutral detergent fibre content (NDF), number of Lactobacilli (Lactobacilli), number of coliforms (coliforms), *Enterobacteriaceae* species richness as determine by DGGE (S_{ent}), *Enterobacteriaceae* species diversity (H_{ent}), Eubacteria species richness (S_{eub}) and Eubacteria species diversity (H_{eub}).

4.3 Results

4.3.1 Prevalence of *E. coli* O157 and STEC virulence genes.

Overall farm prevalence of the *E. coli* O157 specific *rfbE* gene was 52% (90%CI=0.36-0.67) (Table 1). Organic dairy (ORG) farms (n=16) had a prevalence of 61% (90%CI=0.42-0.79) and low-input conventional (LIC) (n=9) of 36% (90%CI:0.15-0.61). Surprisingly, there was no single organic herd where none of the four genes (*rfbE, Stx1, Stx2, eaeA*) were detected and only two LIC herds tested negative for all four genes. The presence of the various virulence genes and the combinations in which they occurred varied widely among the different farms, also among *rfbE* positive herds. Variation was higher within the ORG herds. The majority of the toxin positive farms (13/16 ORG and 7/9 LIC) tested positive for both toxin genes (8/13 ORG and 7/7 LIC); only 2 (ORG) farms tested positive for only *stx1* and 3 (ORG) farms tested positive for only *stx2*. Nine herds were positive for all three virulence genes *eaeA*, *stx1* and *stx2* (5/16 ORG, 4/9 LIC), of which 6 (4/16 ORG, 2/9 LIC) were also positive for *rfbE*. With the exception of one, farms positive for *eaeA* (6 ORG, 4 LIC) were positive for both toxin genes. Seven farms (6 ORG, 1 LIC) were positive for *rfbE* but not for *eaeA*. No statistical differences between ORG and LIC herds were observed with respect to prevalence of virulence genes and the number of virulence genes present per farm.

Table 1. Prevalence (+ indicates presence as determined by Taqman PCR) of the *rfbE* gene (*E. coli* O157) and STEC virulence genes *stx1, stx2* and *eaeA* among 16 organic (ORG) and 9 low-input conventional (LIC) dairy herds. Prevalence and associated 90% confidence intervals are based on the binomial distribution, modelled in @Risk software.

Gene description Farm type	*rfbE* *E. coli* O157	*stx1* Shiga-toxin 1	*stx2* Shiga-toxin 2	*eaeA* intimin
ORG	+			
ORG	+		+	
ORG	+	+	+	+
ORG			+	
ORG	+	+	+	+
ORG		+	+	+
ORG	+			
ORG	+	+	+	
ORG		+		
ORG	+	+	+	+
ORG	+		+	
ORG	+	+	+	+
ORG		+	+	
ORG	+			
ORG		+		+
ORG		+	+	
Prevalence	0.61	0.61	0.67	0.39
90% CI	0.42 - 0.79	0.42 - 0.79	0.48 - 0.83	0.21- 0.58
Modus nr pos. samples*	3	1	3	1
LIC		+	+	+
LIC		+	+	
LIC	+	+	+	+
LIC				
LIC	+	+	+	
LIC				
LIC		+	+	
LIC	+	+	+	+
LIC		+	+	+
Prevalence	0.36	0.73	0.73	0.45
90%CI	0.15 - 0.61	0.49 - 0.91	0.49 - 0.91	0.22 - 0.70
Modus nr pos. samples*	3	1	2	2
Total prevalence	0 .52	0.67	0.70	0.41
90%CI	0.36 - 0.67	0.51 - 0.81	0.55 - 0.84	0.26 - 0.56
Modus nr pos. samples*	3	1	2	1

* Modus of the number of positive pooled samples (maximum is 3)

4.3.2 Chemical and biological characterization of the manure

Several chemical and biological characteristics of the manure were found to be different between ORG and LIC manure (Table 2). Nitrate, total nitrogen, total soluble organic nitrogen, total soluble organic carbon and number of Lactobacilli were significantly higher in the low-input manure (respectively p=0.003, p=0.009, p=0.005, p=0.011, p=0.002, p=0.046). Fibre content (ADF and NDF) was higher in ORG manure (both p=0.046).

4.3.3 Risk factors for the presence of *E. coli* O157 specific *rfbE* and STEC virulence genes

The species richness of the *Enterobacteriaceae* (S_{ent}) was significantly lower in those manures that were positive for *E. coli* O157 (S_{ent}=12±4) compared to the manures tested negative for *E. coli* O157 (S_{ent}=18±7) (p=0.009). Similarly, although not significant, the presence of *eae, stx1* and *stx2* was associated with a lower species richness of eubacteria and *Enterobacteriaceae* as well as lower Shannon indexes. The species richness (expressed as the number of DGGE bands) was positively correlated with the microbial diversity (expressed as the Shannon index) for both eubacteria (r=0.91 p=0.000) and the *Enterobacteriaceae* (r=0.79 p=0.000). With respect to the chemical composition of the manure, total dissolved organic carbon and total nitrogen content was higher in manure positive for *stx2* (p=0.048 and p=0.032).

4.3.4 Survival of GFP-expressing *E. coli* O157:H7: model performance

All decline curves showed nonlinearity, with the presence of shoulders and/or tailing. Clearly, fitting a model based on first-order decline kinetics was not appropriate. The biphasic model was found to be flexible enough to fit all survival curves: fitting to the biphasic model resulted in good fits with an average RMSE of 0.34±0.11 and average R^2 of 0.97±0.01. In addition, observed and fitted values were highly correlated (r = 0.99, p < 0.00) (Fig. 1). The initial decline rate (k_{max1}) was positively correlated with the second phase decline rate (k_{max2}) (r=0.78, p=0.000). The time necessary to reach the detection limit (*ttd*), was strongly correlated to *sl* (r=0.67, p=0.001), k_{max2} (r=0.79, p=0.000) and to a lesser extent with k_{max1} (r=0.49, p=0.017).

4.3.5 Survival in organic versus low-input-conventional manure

The decline of the introduced *E. coli* O157:H7 population was preceded by an initial shoulder of approximately 11 days which did not differ between ORG and LIC manure

Table 2. Average values and their standard deviations of measured chemical and biological characteristics of organic (ORG, n=16) and low-input conventional manure (LIC, n=9).

Type	Chemical characteristics (mg/kg)									Functional groups (log CFU)		Species richness / diversity			
	pH	DM[1]	NO_3[2*]	NH_4[3]	N_{total}[4*]	DON[5* *]	DOC[6*]	ADF[7*]	NDF[8*]	Lactobacilli[*]	Coliforms	S_{ent}[9]	H_{ent}[10]	S_{eub}[11]	H_{eub}[12]
ORG mean	6.7	11.4	7.6	238	897	661	7637	31.8	42.1	5.9	7.3	14	0.98	16	0.85
ORG stdev	0.5	1.8	1.1	104	194	177	2127	3.2	4.6	0.5	0.9	5	0.17	8	0.25
LIC mean	6.8	12.4	9.1	193	1145	918	9377	27.0	37.8	6.6	7.7	15	1.05	19	0.89
LIC stdev	0.4	1.5	0.3	94	218	227	1402	2.5	4.7	0.4	0.6	6	0.14	11	0.26

[*] = significant difference between organic and low-input conventional manure, $p<0.05$.

[1] dry matter, [2] nitrate, [3] ammonium, [4] total nitrogen, [5] dissolved organic nitrogen, [6] dissolved organic carbon, [7] acid detergent fibre, [8] neutral detergent fibre, [9] species richness *Enterobacteriaceae,* [10] species diversity *Enterobacteriaceae*, [11] species richness Eubacteria, [12] species diversity Eubacteria.

Table 3. Mean values and standard deviations of the estimated parameters of the biphasic Geeraerd model and the Augustin model for Survival of *E. coli* O157:H7 GFP in organic (ORG n=16) and low-input conventional manure (LIC n=9) .

		k^{max1}*	k^{max2}	f*	sl	N(0)	ttd*	R2	RMSE
		day^{-1}	day^{-1}	N1/N	days	Log CFU	days		Log CFU
Mean	ORG	0.32	0.06	0.999940	11.3	6.7	109	0.98	0.29
	LIC	0.23	0.03	0.999994	10.2	6.6	94	0.96	0.41
St. dev.	ORG	0.02	0.02	1.1x10-4	9.1	0.2	11	0.01	0.10
	LIC	0.07	0.01	3.41x10-6	8.3	0.2	14	0.02	0.08

* Significantly different between organic and conventional ($p<0.05$)

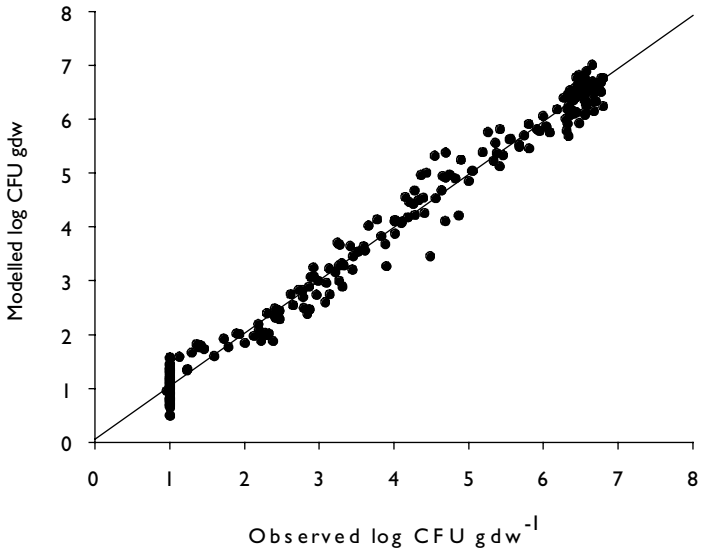

Fig. 1. Correlation between *E. coli* O157:H7 densities in manure (log CFU gdw-1), resulting from fitting survival data to the biphasic Geeraerd model, and observed densities (n=253, r=0.99). The solid line represents the isocline where fitted values are equal to observed values.

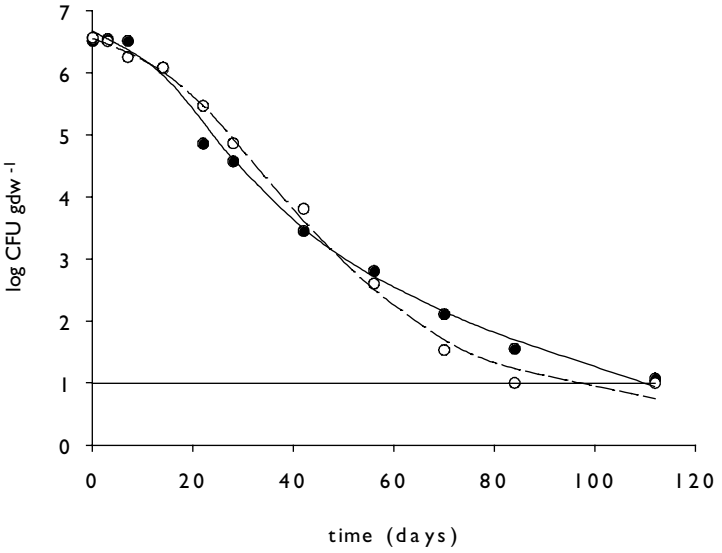

Fig. 2. Average course of decline in organic (solid line, closed circle) and low-input conventional manure (dashed, open circle) as fitted by the biphasic Geeraerd model. Standard deviations are omitted for clarity (average 0.61 for organic and 0.52 for low-input conventional). The horizontal line represents the detection limit.

(Table 3). The initial decline rate was significantly higher for ORG manure (p=0.022). The fitted survival curves of *E. coli* O157:H7 in ORG and LIC manure crossed after approximately 55 days (Fig. 2), caused by an earlier onset of tailing on ORG manure. This coincides with a lower k_{max2} and a significant larger fraction of cells (1-*f*) showing a reduced decline rate (k_{max2}) relative to the fraction showing the initial decline rate k_{max1} (Table 3). The overall survival (*ttd*) was shorter for LIC manure (96±11 days) compared to ORG manure (109±14 days) (p=0.026) (Table 3, Fig. 2).

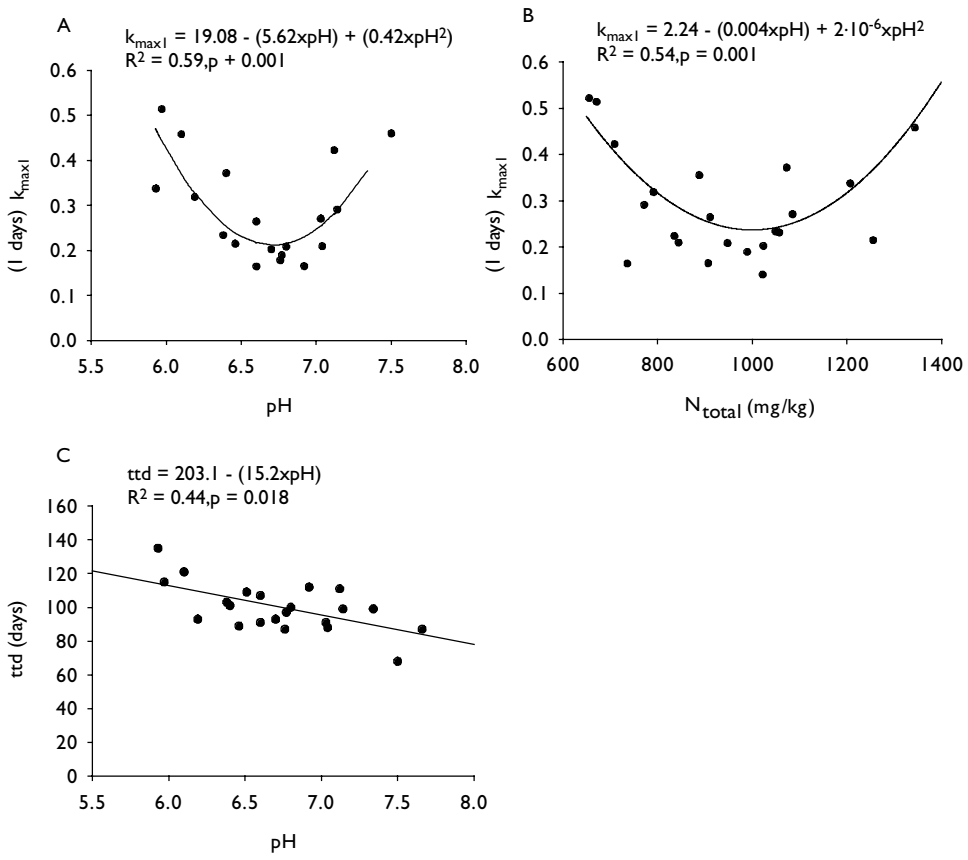

Fig. 3. Observed points (♦) and relations (solid line) between chemical manure characteristics and parameters of the biphasic model: A) the initial decline rate kmax1 and pH, B) kmax1 and total nitrogen content, C) the time needed to reach the detection limit ttd and pH.

4.3.6 Relations survival parameters and chemical manure characteristics

The initial decline rate (k_{max1}) was related to the pH of the manure in the form of an optimum curve and best described with a quadratic relation (Fig. 3). By taking the derivative of this function a pH optimum of 6.71 was determined. By conducting linear regression separately on the data points left and right of the optimum (with fixed intercept) it was observed that $k1_{max}$ decreased with -0.34·pH with increasing pH at values below the optimum, and increased with 0.40·pH with increasing pH at values above the optimum. Thus, the effect of pH on the decline rate was stronger at pH-values above the optimum of 6.7. Both *sl* and *ttd* were negatively correlated with the pH of the manure (respectively r=-0.63, p=0.001 and r=-0.60, p=0.002) (Table 4, Fig. 3). A similar optimum curve in the form of a quadratic relation was observed between k_{max1} and the total nitrogen content (R^2=0.54 p=0.001, optimum at 1000 mg N/kg) (Fig. 3) and k_{max2} and the total nitrogen content (R^2=0.42 p=0.006, optimum at 952 mg N/kg). A negative correlation was observed between $k1_{max}$ and the nitrate content (r=0.48 p=0.029) (Table 4). A linear trend (p<0.1) was observed between *sl* and the ammonium (NH_4) content.

4.3.7 Relations survival parameters and microbial community in manure

The initial decline rate k_{max1} was significantly lower in those manures which were positive for *stx1* (p=0.023), and the fraction of introduced cells which were characterized by a relatively slower decline rate k_{max2} was significantly lower in those manures positive for *stx2* (p=0.04). Both *sl* and *ttd* were negatively correlated with the number of coliforms (Table 4, Fig. 4). Both the decline rates $k1_{max}$ and $k2_{max}$ were negatively correlated with the species richness of the *Enterobacteriaceae* (S_{ent}), which was defined as the number of detected DGGE bands (Table 5). However, quadratic fits were explaining more variation (Fig. 4).

The goodness of fit statistic R^2 and RMSE of the biphasic model, which can be seen as a measure of the predictability of the model, were negatively correlated with the number of coliforms (r=-0.56 p=0.005 and r=0.52 p=0.01) present in the manure. This means a better predictability of the decline of *E. coli* O157:H7 in manure with the biphasic Geeraerd model when less coliforms and copiotrophic bacteria are present. Indeed, as mentioned earlier, the number of coliforms was lower for organic manure and the predictability of decline was better for organic manure.

Table 4. Correlations between model parameters and chemical/biological manure characteristics.

	k_{max1} [1]	k_{max2} [2]	*sl* [3]	*ttd* [4]
pH			-0.63**	-0.59**
NO_3 [5]	-0.48*			
Coliforms			-0.52*	-0.42*
S_{ent} [6]	-0.58**	-0.42**		

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

[1] Decline rate of major sensitive subpopulation, [2] decline rate of minor more resistant subpopulation, [3] shoulderlength, [4] time to reach the detection limit, [5] nitrate, [6] species richness *Enterobacteriaceae* as determined by DGGE.

Table 5. Multiple regression equations with de parameters of the biphasic survival model as dependent variables and the biological and chemical characteristics of the manure as independent variables.

Dependent	Regression equation	Model significance	Model R^2
k_{max1} =	0.53 [intercept, p<0.001] - 0.02 × S_{ent} [a] [R^2_{part}=0.33, p=0.016] + 0.006 × NDF[b] [R^2_{part}=0.13, p=0.073) - 0.11 × stx1[c] [R^2_{part}=0.12, p=0.104] - 0.003 × pH[2] [R^2_{part}= 0.10, p=0.073]	p=0.006	0.69
k_{max2} =	0.23 [intercept, p=<0.001] - 0.02 × coliforms[d] [R^2_{part}=0.35, p=0.010] – 0.003 × Sent[a] [R^2_{part}=0.20, p=0.022) - $9.55 \cdot 10^{-4}$ × ADF[e] [R^2_{part}=0.07, p=0.139]	P=0.003	0.61
sl =	71.58 [intercept, p<0.001] – 7.86 × coliforms[d] [R^2_{part}=0.70, p<0.001]	P<0.001	0.70
ttd =	190.48 - 11.70 × coliforms[d] [R^2_{part}=0.51, p=0.001] - 0.14 × pH [R^2_{part}=0.09, p=0.063]	P=0.007	0.60

[a] Species richness *Enterobacteriaceae* as determined by DGGE

[b] Neutral detergent fibre content: cellulose, hemicellulose and lignin content as percentage of organic matter (% organic matter as NDF per unit dry weight)

[c] Natural presence of the gene coding van Shiga-toxin 1 as detected with Taqman PCR

[d] Total number op coliform bacteria (log CFU / gram dry weight)

[e] Acid detergent fibre content: cellulose and lignin content as percentage of organic matter (% organic matter as ADF per unit dry weight)

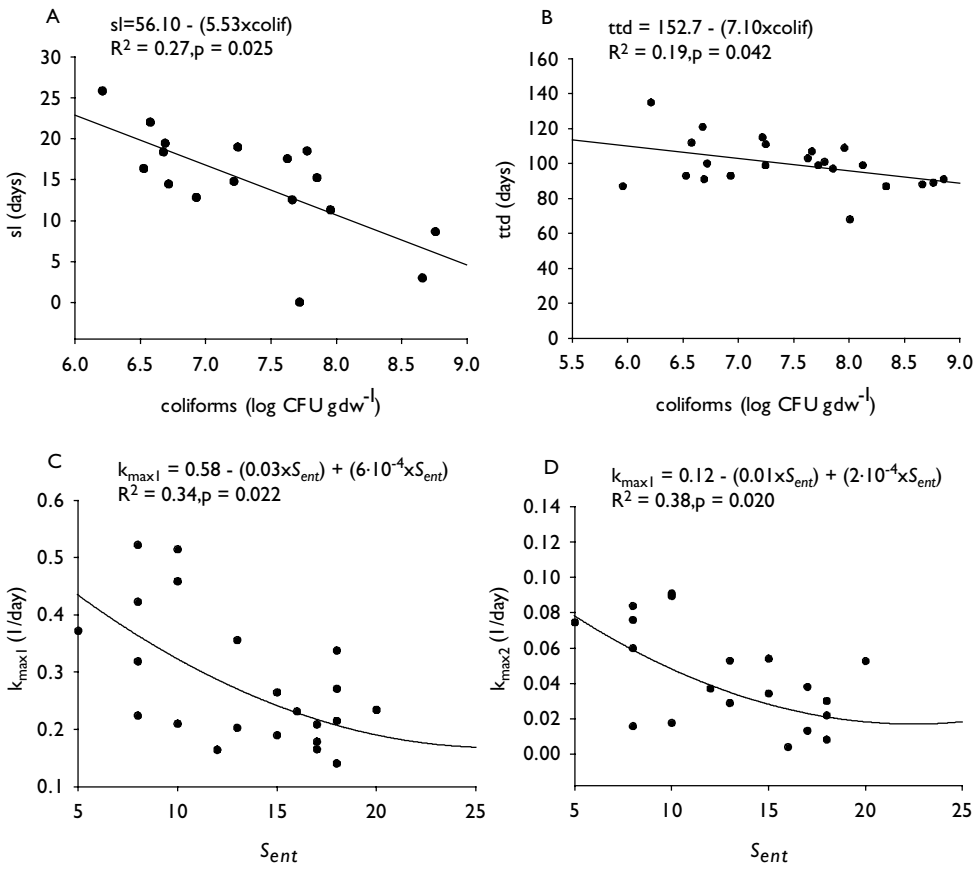

Fig. 4. Observed points (♦) statistical relations (solid line) between biological manure characteristics and parameters of the biphasic model: A) the length of the initial shoulder sl and the number of coliforms, B) time needed to reach to detection limit ttd and the number of coliforms, C) the initial decline rate k_{max1} and the Enterobacteriaceae species richness and E) the second phase decline rate k_{max2} and the Enterobacteriaceae species richness.

4.3.8 Multiple regression explaining parameters

Multiple regression equations were constructed to relate the parameters of the survival models to chemical and biological characteristics of the manure. This resulted in significant models for k_{max1}, k_{max2}, *sl* and *ttd* with relatively high predictive value, i.e. high R^2 (Table 5). The initial decline rate k_{max1} could mainly be predicted by the species richness of the *Enterobacteriaceae* (negative relation) and the presence of the gene coding for Shiga-toxin 1 (negative relation). The remaining variation could be explained by the fibre content (positive relation) and the pH (quadratic relation). Decline rate k_{max2} could be explained by the total number of coliforms (negative

relation), the species richness of the *Enterobacteriaceae* (negative relation) and the fibre content (positive relation). Interestingly, k_{max1} increased with increasing fibre content while k_{max2} decreased with increasing fibre content. The length of the initial shoulder of the decline curve was determined solely by the total number of coliforms present in the manure (negative relation). The overall rate of decline, measured as the time to the detection limit *ttd*, was also best explained by the number of coliforms (negative relation) in the manure and the pH (negative relation) (Table 5). In turn, the level of coliforms were predominantly determined by the pH of the manure, with increased number of coliforms with increasing pH (R^2=0.36, p=0.011). With respect to the model, variation in *ttd* was best explained by the variation in the shoulderlength *sl* (partial R^2 = 0.51).

4.3.9 Extremes and farming styles

The four best *E. coli* O157:H7 supporting manures (i.e four highest *ttd* values; all ORG) were derived from farms with exclusively Frisian Holstein cows while two of the four farms from which the manure supported the worst survival of *E. coli* O157:H7 (2 ORG and 2 LIC) harboured another breed (both ORG) next to Frisian Holsteins. In addition, the four manures that best supported survival of *E. coli* O157:H7 had significantly higher number of naturally present STEC virulence genes (p=0.03), higher levels of NO_3, higher levels of dissolved organic carbon (DOC) (p=0.01) and lower levels of coliforms p=0.001).

4.4 Discussion

4.4.1 Prevalence of the *E. coli* O157 specific *rfbE* gene.

The survey conducted in this study showed that the *rfbE* gene, which is characteristic for *E. coli* O157, was present at 52% of 25 Dutch dairy farms sampled in August and September 2004. This is relatively high compared to the prevalence at Dutch dairy farms in a previous study, namely 7.2% out of 678 farms (48). The same sampling strategy was used but in the present study the *E. coli* O157 specific *rfbE* gene was detected by real-time PCR instead of the detection of *E. coli* O157 cells with immunomagnetic separation, which could lead to different results. In addition, the earlier reported prevalence of 7.2% was an average over 4 years including all seasons while the present study included only the summer of 2004. The first study determining the prevalence of *E. coli* O157 on Dutch dairy farms was, just as the present study, a point estimate in time (fall 1996) and reported a prevalence of 70% (n=10) (25). It is generally known

that *E. coli* O157 prevalence shows seasonality with highest prevalence's in the warmer months (60) and Schouten *et al* showed peaks in prevalence occurring regularly, up to 36% in September 2000 (48). Monthly prevalence over 1999 and 2000 was positively correlated with mean and maximum temperatures of the corresponding months ($r=0.60$, $p=0.002$ and $r=0.53$, $p=0.008$) (48) (2). August and September 2004 were characterized by high average temperatures compared to the long year average (18.8°C versus 17.2°C and 15.2 versus 14.2°C) and by high maximum temperatures (32.5 and 28°C), which make the 52% farm prevalence found in the present study plausible.

In other countries, reported *E. coli* O157 prevalence among dairy farms ranged from 7.1 to 80% (average of 39 ± 27.2, $n=20$), with generally a higher prevalence in the USA (50.4 ± 24.9, 10 studies) compared to Europe (31.8 ± 27.8, 10 studies). It must be stressed that direct comparison of prevalence's across studies is confounded by the use of different sampling and detection methods, environmental conditions and geographical location.

Although prevalence of *rfbE* was higher among certified organic farms (ORG) (61%), this was not statistically different from the low-input conventional farms (LIC) (36%). This trend is identical to a recently published report, where *E. coli* O157 was found in 4 out of 8 organic dairy farms (50%) and in 3 out of 18 conventional dairy farms (16.7%) in the USA (12). In Switzerland *E. coli* O157:H7 showed a herd prevalence of 25% at organic farms and 17% at integrated (conventional) farms (both $n=60$), but this was also not significantly different, and no difference in the risk to carry this pathogen was found between both farm types (35).

4.4.2 Risk factors for the presence of *rfbE*.

The species richness of *Enterobacteriaceae* was significantly lower in those manures which were positive for *rfbE*. Ecological theory argues that, at small spatial scales, diverse communities are more competitive and therefore more resistant to invasion than less diverse communities (16, 32). For example, the invasibility of wheat rhizosphere communities by *Pseudomonas aeruginosa* was inversely related to the level of microbial diversity (38). It has been hypothesized that species richness and/or diversity will increase with increased resource heterogeneity (37) and that a community becomes more susceptible to invasion whenever there is an increase in the amount of unused (available) resources because the invader will encounter less intense competition from resident species (13). With respect to cattle and STEC, diet is a major factor in determining the abundance and nature of nutrient resources in the digestive tract and might therefore be an important factor in controlling the susceptibility to STEC invasion. A higher level of unused available nutrients and/or lower resource heterogeneity may result in a lower microbial diversity and an increased susceptibility

to pathogen invasion. Fewer bovine commensal *E. coli* serotypes were isolated from the faeces of cattle on feedlot/grain diets, compared with cattle on pasture/roughage-based diets (5) and some studies concluded that the shedding of *E. coli* O157:H7 is reduced when are fed a low energy hay-based diet compared to a high energy grain-based diet (23, 47). We observed higher levels of total dissolved organic carbon and total nitrogen in manure positive for *stx2*, which might be an indication for the a possible relation between the nutrient status of the gut/manure and the presence of VTEC virulence factors.

4.4.3 Prevalence and risk factors for the presence of STEC virulence genes

Overall, 80% of the farms tested *stx*-positive. Very high prevalence of Shiga-toxins was also demonstrated for Norway (100%, n=50) and Ohio in the USA (70%, n=50) (36). Quite some organic farms (7/9) were positive for *rfbE* but negative for intimin. This could indicate the presence of less virulent O157-serotypes lacking intimin like described for *E. coli* O157:H7, O157:H8, O157:NM (18) (27). From epidemiological studies it is known that human clinical isolations of STEC showed a significant association between the presence of *eaeA* and *stx2* (6). Our results indicate that although there is a trend of increased prevalence of *rfbE* with ORG farms, more LIC farms were tested positive for all VTEC virulence genes (both *eaeA* and *stx2*). It must be stressed that the detected virulence genes on a single farm do not necessarily originate from one cell or serotype since we did not isolate serotypes. However, given the fact that most STEC virulence markers are on mobile genetic elements, we may assume that the chance of the existence or future emergence of virulent serotypes by horizontal gene transfer, is higher at the LIC farms.

4.4.4 Modelling survival of *E. coli* O157:H7 gfp in manure

The obtained survival curves typically were nonlinear and showing considerable variation. The use of first-order kinetics and log-linear modelling of survival was clearly not appropriate. Survival of human pathogens in manure is also often nonlinear, but attempts to model it accordingly are scarce. Recently, the decline of *E. coli* O157:H7 in manure was described with a logistic (20) and an exponential decay model (4). Unfortunately, these relatively simple models were not able to cover the range of survival curves obtained with the present study. The biphasic Geeraerd model was flexible to such extent that all survival curves could be fitted to this model with good accuracy (significant and high R^2). Biphasic inactivation curves, where the initial rate of decline is followed by a slower second phase (tailing), have been observed regularly

within food microbiology (1, 28, 51). Also in environmental substrates, pathogen die-off occurs often in two stages (43). For example, a two-stage die-off model was used to describe the decline of *E. coli* O157 in soil (42).

4.4.5 Manure characteristics affecting the survival of *E. coli* O157:H7.

The survival curves showed initial shoulders in 17 out of the 25 cases, with an average length of 11 days. No differences were observed in shoulderlength (*sl*) between ORG and LIC manure. The variation in *sl* and overall survival time *ttd* were best explained by the variation in the level of coliforms in the manure, with shorter initial shoulders and shorter overall survival with higher numbers of coliforms. Native coliforms may occupy the same ecological niche as *E. coli* O157:H7 (niche exclusion principle) and/or an increase in the level of coliforms may increase the level of competition for available nutrients. In turn, the number of coliforms was predominantly determined by the pH of the manure, with increasing numbers with increasing pH.

The pH of the manure also entered the regression model for k_{max1}. Decline rate k_{max1} showed a quadratic relation with pH, which makes sense since organisms usually show curvilinear relations with environmental variables. We observed an optimum pH of 6.7, which is close to the optimal pH of 6.9 for growth of *E. coli* O157:H7 in broth (41). Higher and lower pH levels, within a range of 5.9 to 7.2, resulted in increased decline rates but the effect of higher pH was stronger than that of lower pH. The decline rate of *E. coli* O157:H7 increased linearly with the pH when survival was studied in manure from cows fed three different diets (20). However, the pH range in which linear increase in decline rate was most evident ranged between 6.8 up to 7.7, which is the same range in which we observed the positive relation between pH and decline rate in the present study. It is known that *E. coli* O157:H7 possesses several systems in order to survive exposure to low pH and can be considered quite acid tolerant (7). It therefore might have a selective advantage at low pH (14). In addition, organic nitrogen might be converted to ammonia at higher pH. It has been demonstrated that ammonia can be toxic to *E. coli* O157:H7 and can cause a significant reduction in numbers (26). A significant reduction of *E. coli* O157:H7 was also achieved by a combination of high concentrations of carbonate and ammonia which require a high pH (44).

4.4.6 Explaining the nonlinear and biphasic nature of survival curve.

The vitalistic concept proposes that in a genetically homogeneous population, phenotypic variation in physiological responses exists such that resistance to a lethal agent is not uniform (28). Subsequently, the nonlinearity of the survival curve reflects the inactivation of a population of cells which is heterogeneous with respect to decline

kinetics (45, 57). The biphasic model used in the present study assumes the existence of two subpopulations, each with its own inactivation characteristics. Theoretically, tailing occurs because the relative size of the more resistant subpopulation and the associated weight of k_{max2} then increases which results in a net slower decline and tailing. The earlier onset of tailing in ORG manure can therefore be explained from the significantly higher fraction of *E. coli* O157:H7 in the more resistant subpopulation.

Although the overall decline curve could be explained as the cumulative distribution of an underlying distribution of decline kinetics, the differences in decline kinetics between the different manures are difficult to explain from innate differences among *E. coli* O157:H7 cells within the starting culture. We propose that the nonlinear survival pattern is the result of a changing competition pressure over time due to a changing nutrient availability. Likewise, differences in decline kinetics between manures can be explained from differences in the substrate composition and differences in the changes over time. During the first days after inoculation *E. coli* O157:H7 uses the easily available nutrients and maintains equilibrium between growth and death within the population. When the easily available nutrients become limited, this equilibrium is disturbed and the overall death rate of the population will exceed the overall growth rate, resulting in a decline phase. Additionally, the manure in the pots will become more anaerobic over time and *E. coli* O157:H7 has to switch from aerobic to anaerobic metabolism. The levels of easily available nutrients are usually lower in manure with higher fibre content. Possibly, the higher fibre content and lower levels of total soluble organic carbon and nitrogen of ORG manure resulted in an increased initial nutrient stress for *E. coli* O157:H7 which resulted in a faster initial decline. The more abundant easily available nutrients in LIC manure became limited after 40-50 days while the higher fibre content of ORG manure resulted in continued release of nutrients as a result of the decomposition of complex cellulose and lignin structures by cellulolitic organisms. As a result, the average survival curve for ORG manure levelled of and crossed the average curve for LIC manure after approximately 50 days. Indeed, according to the multiple regression analysis the initial decline rate was increased with higher fibre content while the decline rate of the second phase was decreased with higher fibre content. Earlier obtained results showed a significant faster overall decline of *E. coli* O157:H7 in manure from dairy cattle placed on diets differing in fibre content (20). However, the fibre content of the manures resulting from the high fibre diet (pure straw) was significantly higher (ADF=50% and NDF=66%) than the fibre contents of ORG and LIC manure in the present study (Table 2). Probably, other limiting factors overrule the advantage of a slow but continuous nutrient gift from the fibre polymers at very high fibre contents. One such factor might be the pH, which was substantially higher with the high fibre manure (7.4) compared to the ORG manure in the present study (6.7, Table 2).

4.4.7 Additional factors explaining variation in survival

In the present study no effect of lactobacilli on the natural presence *E. coli* O157 *rfbE* gene and the survival of GFP-*E. coli* O157:H7 was observed. Certain strains of *Lactobacillus* exert growth inhibition of *E. coli* O157 and/or bactericidal activities on *E. coli* O157:H7 (9). Supplementation of cattle with *Lactobacillus acidophilus* NP51 resulted in significant lower prevalence of *E. coli* O157 among experimental cattle herds (63). Probably, the inhibitory effect of lactobacilli is limited to certain strains and even varies with different *E. coli* O157 strains. This might be related to our finding that among the four manure types which were the worst supportive to *E. coli* O157 two were derived from farms where in addition to Frisian Holstein cows another breed was present. It could be that some specific antagonistic bacteria were present in the manure from these more rare cattle breeds. Moreover, the lack of a relation between survival and the number of lactobacilli might also have to do with the fact that the conditions in the manure were not anaerobic, while most lactobacilli favour anaerobic conditions when sugars are fermented to lactic acid.

4.4.8 Conclusions

Although the prevalence of the *E. coli* O157 specific *rfbE* gene was higher at ORG farms compared to LIC farms, relatively more LIC farms tested positive simultaneously for STEC virulence genes *eaeA*, *stx1* and *stx2*. The species diversity of *Enterobacteriaceae* was higher in manure positive for *rfbE*, indicating the possible important role of the microbial diversity in STEC epidemiology. The biphasic decline model was flexible enough to describe all 25 obtained survival curves with good fits. Initial shoulderlength and overall survival were determined by and negatively related with the numbers of coliforms present in the manure and the pH. It is proposed that the nonlinear survival pattern is the result of a changing competition pressure over time due to a changing nutrient availability, instead of the presence of subpopulations differing in the level resistance.

Further research should focus on isolating and serotyping of STEC strains on organic and conventional farms in order to investigate whether different populations of STEC are present on organic farms compared to conventional farms. In addition, a detailed understanding of the interaction between pathogen, the nutrient status of the substrate and the native microbial community is needed in order to develop control strategies that reduce pathogen survival and thus spread to the environment and the food chain.

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Chapter 5

Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils

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Abstract

The recent increase in foodborne disease associated with the consumption of fresh vegetables stresses the importance of the development of intervention strategies that minimize the risk of preharvest contamination. To identify risk factors for *E. coli* O157:H7 persistence in soil we studied the survival of a Shiga-toxin deficient mutant in a set of 36 Dutch arable manure-amended soils (organic/conventional, sand/loam) and measured an array of biotic and abiotic manure-amended soil characteristics. The Weibull model, which is the cumulative form of the underlying distribution of individual inactivation kinetics, proved to be a suitable model for describing the decline of *E. coli* O157:H7. The survival curves generally showed a concave curvature, indicating changes in biological stress over time. The calculated time to reach the detection limit *ttd* ranged from 54 to 105 days and the variability followed a logistic distribution. Due to large variation among soils of each management type, no differences were observed between organic and conventional soils. Although the initial decline was faster in sandy soils, no significant differences were observed in *ttd* between both sandy and loamy soils. With sandy, loamy and conventional soils the variation in *ttd* was best explained by the level of dissolved organic carbon per unit biomass carbon *DOC/biomC*, with prolonged survival at increasing *DOC/biomC*. With organic soils the variation in *ttd* was best explained by the level of dissolved organic nitrogen (positive relation) and the microbial species diversity as determined by DGGE (negative relation). Survival increased with a field history of low-quality manure (artificial fertilizer and slurry) compared to high-quality manure application (farmyard manure and compost). We conclude that *E. coli* O157:H7 populations decline faster under more oligotrophic soil conditions, which can be achieved by the use of organic fertilizer with a relatively high C/N ratio and consequently a relatively low rate of nutrient release.

5.1 Introduction

During the last three decades, an increasing number of outbreaks caused by foodborne pathogens has been associated with the consumption of fresh produce (21, 51). Recently, a large multi-state outbreak of *E. coli* O157:H7 due to the consumption of contaminated fresh spinach in the USA resulted in 187 cases of illness (including 97 hospitalizations and three deaths) (3). Produce can become contaminated at any point during the primary production, processing and distribution. For pathogens with animal reservoirs, like VTEC *E. coli* O157:H7, contamination in the field can occur when contaminated manure is used for fertilization. *E. coli* O157:H7 may become associated with the surface of vegetables grown in contaminated manure-amended soil (23, 42) where they can survive and even grow (1). Recently it has been demonstrated that *E. coli* O157:H7 can become internalized in the plant during growth in contaminated soil (15, 54), which may constitute a public health risk since these bacteria are unlikely to be removed during post-harvest sanitation or washing by consumers.

Prevention of preharvest contamination of fresh produce is an essential part of a systems approach focused on applying interventions designed to achieve delivery of microbiologically safe produce to consumers (5). Suppression of human pathogens in manure-amended soil and the subsequent prevention of spread into the food chain by contamination of produce must be realized by intrinsic factors of the soil system since anti-bacterial pesticides applicable to soil are not available. In order to develop strategies that minimize the risk of pathogen survival and spread within the agricultural system and food chain, it is important to study the fate of *E. coli* O157 in environmental substrates like manure-amended soil and to understand how manure-amended soil conditions affect its survival (21).

Reported survival times of *E. coli* O157:H7 in manure-amended soil range between several weeks and more than 6 months, depending on soil type, bacterial strain and experimental setup (4, 23, 26, 27, 43). Both abiotic (temperature, pH, soil moisture, soil type) and biotic (composition and diversity of the microbial community) factors affect the survival capabilities of bacteria introduced into the soil habitat (61). Most studies considered the effects of soil characteristics independently. Since the extent to which these factors affect survival most likely depends on interactions between the various environmental factors, the overall set of abiotic and biotic soil characteristics should be taken into account.

Only few attempts have been made to link survival of *E. coli* O157:H7 with soil physico-chemical and biological variables (26, 40, 44). Extrapolating results from studies conducted with commensal *E. coli*, which is often used as an indicator organism for faecal contamination, seems not entirely valid since these indicator organisms behave differently and may survive longer than *E. coli* O157:H7 (10, 13, 40). Moreover, the

existence of naturalized soil-borne *E. coli* in densities up to 10^3 cfu/g confounds the use of this bacterium as a reliable indicator organism for pathogen spread and survival (22).

Soil management can significantly affect soil characteristics and soil functioning (7, 60). While in conventional arable farming systems soil fertility is maintained with the use of synthetic fertilizers next to the use of organic amendments (e.g. manure), organic farmers refrain from the use of synthetic fertilizers and soil fertility is built up solely with organic amendments (frequently consisting of animal manure). Organically managed soils generally show higher microbial diversity, total of microbial activity and microbial biomass compared to conventional soils (35, 60). These differences have been associated with an enhanced suppression of soil-borne (fungal) plant pathogens (59). Currently it is not known to which extent various soil management practices can influence the survival of human enteric pathogens, introduced by the amendment of soil with manure.

Recently, 12 pairs of neighboring organic and conventional agricultural soils in the Netherlands were compared with respect to biological and physico-chemical soil properties (60). In the present study 18 pairs of organic and conventional soils, including the above mentioned 12, were used to determine differences in decline kinetics of *E. coli* O157:H7 in a wide array of soils. More specifically, the goals were to (i) model the survival of *E. coli* O157:H7 in a range of soils with an appropriate inactivation model and quantify the variability in survival, (ii) determine the relative importance of management type and soil type with respect to the survival of *E. coli* O157:H7 and (iii) identify physical, chemical and/or biological manure-amended soil characteristics which are responsible for differences in decline kinetics.

5.2 Materials and methods

5.2.1 Soil collection

Soil was collected from six SKAL (inspection body for organic production) accredited organic (coded ORG) farms with lettuce fields throughout the Netherlands, representing to the best of our knowledge all lettuce producing organic farms in this country. Six conventional (coded CONV) farms with lettuce fields were selected as much as possible on comparable soil types, so that pairs of ORG and CONV farms could be formed. In addition, 12 SKAL accredited ORG farms with other crops were selected throughout the Netherlands (60). For each ORG farm a neighbouring CONV farm was selected with an adjacent field, identical soil type and with the same crop (potato, grassland, sugar beet, wheat or maize). Each pair of ORG and CONV farms was sampled on the same day. Throughout each sampled field, 10 soil sub-samples (20 cm deep) were collected between

the plants with an augur and mixed. Crop coverage at the moment of sampling was low on all 24 soils because of the low temperatures in early spring. Samples were collected during March, April and May 2005. All samples were transported to the laboratory in plastic bags, thoroughly mixed, sieved through 0.5 cm mesh to remove plant parts and earthworms and stored at 5 °C until the start of the experiment (May 2005).

5.2.2 Soil texture

Fractions of clay particles (<2 μm), silt particles (2-50 μm) and sand particles (50-2000 μm) were determined in all collected soils before mixing with manure by laser diffraction. Soils were classified into soil types according to a US texture diagram and summarized as being sand or loam.

5.2.3 Adjusting water content

To ensure similar water availability in the different soils the water content of each individual soil was adjusted to 60% of its maximum water holding capacity (WHC). The actual water content was measured by drying approximately 5 g soil for 24 h at 105°C. The WHC of the soil was determined by adding an excess of distilled water to approximately 50 g of field-moist soil sample. The sample was left overnight covered with aluminium foil to prevent evaporation. The well-drained soil was filtered in a funnel with filter paper mounted on a collecting flask and again allowed to stand overnight covered with aluminium foil. Subsequently, the WHC was determined by drying the soil sample of approximately 5 g of well-drained soil for 24 h at 105°C. The amount of water to be added was calculated by taking 60% of the WHC minus the water content of the field-moist sample. From each collected soil 1000 g was adjusted to a water content of 60% of the WHC. Subsequently, two portions of 450 g from each soil were placed in plastic bags. One portion was mixed with non-inoculated manure and used as a control on which all chemical and biological characteristics were determined (all at start of the experiment) and one portion was mixed with inoculated manure to study pathogen survival.

5.2.4 Chemical characterization

Dried (24 h at 40°C) soil-manure samples from the control pots were ground, sieved through a 2 mm mesh and analyzed at the start of the experiment. The pH was measured in water with an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO_3), ammonium (NH_4) and total dissolved nitrogen (N_d) content were determined colorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York). Dissolved organic nitrogen (DON)

was calculated as the difference between N_{ts} and the amount of nitrogen present as NH_4^+ and NO_3^-. Dissolved organic carbon (DOC) was measured by a carbon analyzer in a soil extract of 0.01 M $CaCl_2$. Total carbon (C_{total}) and total nitrogen (N_{total}) were measured by the Dumas method followed by detection by a Fisons element analyzer type EA 1108 (Therom Finnigan Italia S.P.A., Milan, Italy).

5.2.5 Copiotrophic and oligotrophic bacteria enumeration

Two samples from each control pot of approximately 1 g were suspended in 4.5 ml buffered peptone water (BPW), vortexed vigorously for 1 minute, sonicated in an ultrasonic cleaner (Bransonic 12, Branson Cleaning Equipment Co., Shelton, CT) for 30 s, vortexed again for 30 s and serially diluted in BPW. Fifty microliters of suitable dilutions were plated in duplicate on high and low carbon medium for quantification of respectively copiotrophic and oligotrophic bacteria (49). The high carbon medium contained 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KNO_3, 1.3 g $K_2HPO_4 \cdot 3H_2O$, 0.06 g $Ca(NO_3)_2 \cdot 4H_2O$, 25 g glucose, 2 g enzymatic casein hydrolysate (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) and 17.0 g Agar no. 3 (Oxoid Limited, Basingstoke, UK) per liter. The low carbon medium was similar but with 1000 fold diluted carbon concentration. After incubation for 60 h on high-C medium (for copiotrophic bacteria) and 3 weeks on low-C medium (for oligotrophic bacteria), bacterial colonies were counted and colony-forming units (CFUs) were calculated per g dry soil.

5.3.6 Basal respiration

Microbial activity was assessed by measuring the basal respiration rate. Basal respiration of the manure-amended control soils was determined in duplicate with an automated system in which a continuous air-flow of 65 ml/min was led over 60-90 g of fresh weight soil in glass tubes (length 24 cm, diam. 3.5 cm) incubated at 20 °C for 24 h. After passing over the soil, the moisture in the air was absorbed by special granulate and the CO_2-concentration was analyzed by means of a computer-controlled switching device and an infrared CO_2 analyzer (ADC 7000 analyser, Analytical Development Corporation, Hoddesdon, UK). Two empty tubes were used as controls to measure the concentration of CO_2 in the air. For calculation of the basal respiration the readings of the first 10 h of incubation were omitted. The respiration was expressed in μg CO_2/g dry weight/h.

5.2.7 Microbial biomass

Microbial carbon present in the manure-amended control soil was measured using the fumigation extraction method (62). The method is based on the assumption that

the organic carbon measured after a 24 h $CHCl_3$-fumigation originates from the cells of the microbial biomass so that the difference between organic carbon extracted by 0.5 M K_2SO_4 from a fumigated and a non-fumigated sample of the same soil can be used to estimate soil microbial biomass. We used 2.22 instead of 2.64 as a factor to convert the carbon released by fumigation into biomass carbon since we used UV-persulphate as the analytical procedure to measure organic carbon instead of dichromate digestion (65). Microbial biomass was expressed as mg C / kg dry weight soil-manure mixture.

5.2.8 DGGE

DGGE analyses were conducted in order to relate the survival of *E. coli* O157:H7 to the species richness and species diversity of the microbial population present in the manure-amended soil. DNA was extracted from 300 mg (fresh weight) manure-amended soil with the Bio101® Systems FastDNA® SPIN® Kit for Soil according to the manufacturer's specifications (Qbiogene, Inc., Carlsbad, CA, USA) except that bead beating (three times 90 s) was used instead of the FastPrep1 instrument. The 16S rRNA gene of eubacteria were amplified from soil-manure DNA with the eubacterial primer pair U968-GC and L1401 (12). The eubacterial PCR was performed using a touchdown scheme for 30 thermal cycles and finished by an extension step at 72°C for 30 min (25, 48). The PCR products were examined by standard 1.2 % (w/v) agarose-0.5x Tris-borate-EDTA (TBE) gel electrophoresis with ethidium bromide staining, to confirm product integrity and size. DGGE was performed using the DCode system (Bio-Rad Laboratories, Hercules, CA, USA). We used 6% acrylamide gels (37.5 acrylamide:1 bisacrylamide) with a 45–60% denaturing gradient (41) to separate the generated amplicons (100% denaturant is 7 M urea and 40% formamide) and an 8% acrylamide stack without denaturing agents. The gels were poured from the top in the DCode template, prepared with Gelbond PAG film (Amersham Pharmacia Biotech AG, Uppsala, Sweden) to one side, using a gradient maker and a Heidolph Pumpdrive (Heidolph, Schwabach, Germany) set at 4 ml/min. Eubacterial PCR products derived from DNA of each manure sample were loaded in adjacent slots.

Electrophoresis was performed in 0.5x TAE buffer for 16 h at 100 V at a constant temperature of 60°C. Gels were stained with Bio-Rad's Silver Stain (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol, but using the protocol for gels >1 mm thick instead of 0.5–1 mm to compensate for the barrier formed by the Gelbond. After staining the gels were preserved for at least 1 h in Cairn's preservation solution of 25% ethanol (v/v) and 10% glycerol (v/v), covered by a permeable cellophane sheet (Amersham Pharmacia Biotech Ag, Uppsala, Sweden) and dried overnight at 60°C.

The gels were scanned using ScanSoft Omnipage pro. 14 at a resolution of 300 dots per inch. Scanned gels were analysed with Phoretix 1D (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). Bands were selected manually. Data of different DGGE gels were standardized by referring to the DGGE marker. The 16S rDNA fragments detected by DGGE were considered to represent the most numerous bacterial groups, making up at least 0.1-1% of the total community (41). The bacterial diversity was estimated in two ways: as species richness *S*, and as the Shannon-Wiener index of bacterial diversity, *H*. Species richness *S* was defined as the number of DGGE detected bands per soil type. The Shannon-Wiener diversity index was calculated as $H' = -P_i \log P_i$ based on the relative band intensities as formulated by Eichner *et al.* (11), where P_i (importance probability of the band) is defined as n_i/N where n_i is the area of the peak in intensity and N the sum of all peak areas in the lane profile. The Shannon-Wiener diversity index is a general diversity index which increases with the number of species and which is higher when the mass is distributed more evenly over the species. DGGE analysis was done in duplicate where the replicas were on different gels. *S* and *H* were calculated as the mean of the two replicas.

5.2.9 Strain

Strain *Escherichia coli* O157:H7 B6-914 GFP-91 was kindly provided by Dr. Pina Fratamico (16). This strain does not produce the Shiga-like toxins I or II ($Stx1^-$ $Stx2^-$), but contains the pGFP cDNA vector (Clontech Laboratories, Inc. Palo Alto, CA) expressing green fluorescent protein (GFP) and ampicillin resistance. The use of an GFP-expressing plasmid is legitimate since the presence of the plasmid does not affect the intrinsic characteristics of the strain, no significant behaviour differences were observed between GFP-transformed strains and parent strains and the stability/ expression of the marker in unfavourable conditions was demonstrated (16, 55, 63) . In addition, no difference in survival between toxin positive ($Stx1^+$ $Stx2^+$) and toxin negative ($Stx1^-$ $Stx2^-$) *E. coli* O157:H7 in bovine manure was observed (31). Survival in manure of *E. coli* O157:H7 which passed the intestinal tract of cattle was not different from the survival of the same strain directly inoculated into the manure (Scott 2006). Bacteria were stored at –80°C and checked for viability prior to use by growing on Luria-Bertani medium supplemented with ampicillin (50 μg ml^{-1}).

5.2.10 Inoculation of manure and preparation of soil-manure mixtures

Manure from steers (MRIJ breed mixed with Montbéliarde breed) on a standard 50% grass/clover-silage + 50% dried grass diet was collected from a manure-straw

heap (30-50°C at 20 cm depth) at the organic experimental farm Droevendaal (Wageningen University and Research Centre, The Netherlands). Seventy-two portions of 50 g were weighted and put in small plastic bags.

Bacterial inocula were grown in Erlenmeyer flasks containing 150 ml buffered peptone water (BPW) supplemented with 50 µg/ml ampicillin, followed by incubation at 37 °C on an orbital shaker (200 rev min^{-1}) for 18 h. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times and resuspended in sterile distilled water. The number of cells per ml of suspension was determined using the spectrophotometer, where OD 0.7 at 630 nm was equal to 1×10^9 CFU ml^{-1}. Cells suspended in BPW were added to half of the manure portions with a final density of 1×10^7 CFU per gram dry-weight of final soil-manure mixture, taking into account the water content of the manure and that of the individual soils. An identical volume of BPW was added to the control treatments. The manure and the inoculum were thoroughly mixed by kneading in a plastic bag from the outside by hand. Subsequently, the inoculated portions of manure were added to and thoroughly mixed with the 450 g portions of soil. The mixture was transferred to 1 L pots which were closed (but with the ability of gas exchange) and incubated at 16°C in darkness.

5.2.11 Sampling

The inoculated soil-manure mixtures were sampled nine times within 60 days after inoculation to determine the survival of the pathogens. At each sampling time two samples of approximately 1 g of each replica was removed from the middle of the mixture using a sterile spoon and put in separate pre-weighed dilution tube with 4.5 ml of 0.1% peptone. Sampling holes were closed. Sample-tubes were weighed to determine the exact size of the sample. Samples were vortexed and put in a Branson 5200 ultrasonic bath for 30 s. The samples were vortexed again and 10-fold serial dilutions were made. From the two highest dilutions 50 µl was plated in duplicate on Petri dishes with Sorbitol MacConkey (SMAC, Oxoid) agar supplemented with 50 µg/ml ampicillin (detection limit of 100 CFU/g dry weight). The number of necessary dilutions was estimated based on preliminary counts. Cell suspensions were spread on the surface by shaking with 2 mm sterile glass beads. The inoculated plates were incubated at 37 °C for 18 hours. Numbers of *E. coli* O157:H7 were determined after incubation for 18 hours at 37 °C by counting green fluorescent CFU's using a dark-blue lamp (Philips PL-S 9W/08 Blacklight Blue, peak at 365 nm UV-A). Colony counts were calculated to log number of CFU gdw^{-1}. The detection limit of the plating technique was approximately 100 CFU per gram dry weight of manure-amended soil.

5.2.12 Statistical analysis

Model description

Survival of *E. coli* O157:H7 was modelled by fitting the experimental data to the Weibull survival function (SAS® system for Windows version 8.02, SAS Institute Inc, Cary, NC, USA, 2001). This model is based on the assumption that the cells' resistances to stress, as encountered in the soil-manure mixture, follow a Weibull distribution and that the survival curve is the cumulative form of this underlying distribution of individual inactivation kinetics (Mafart 2002, van Boekel 2002):

$$\log \frac{N}{N_0} = -\left(\frac{t}{b}\right)^n$$

where log N/N_0 is the log number of the relative population size (CFU gdw^{-1}) at time t (days), b (scale parameter) represents the *time of first decimal reduction* (days) and n (shape parameter). For $n>1$ a convex curve is obtained, while for $n<1$ a concave curve is obtained. Model performance was assessed by calculating the regression coefficient (R^2) and the Root Mean Squared Error (RMSE). In addition, the residuals were subjected to a test for normality. The performance of the Weibull model was compared with a linear model. In addition to the model parameters, the time needed to reach the detection limit of 100 CFU gdw^{-1} was calculated (*ttd* in days).

Data analysis

Model parameters, physico-chemical and biological variables were checked for normality and transformed when necessary. Differences in physico-chemical and biological variables between ORG and CONV soils were assessed with paired t-tests. Differences between sand and loam and the four different combinations were assessed by independent sample t-tests. Pearson correlation matrices were constructed to reveal linear relations between model parameters and environmental variables. Multivariate analysis of variance (MANOVA) was conducted in order to reveal the effect of soil and management type on both Weibull model parameters simultaneously. Analyses were conducted in SPSS v 12 (SPSS Inc., Chicago, Illinois, USA).

The variability in the model parameters were described by fitting the values to probability distributions using @Risk software (version 4.5.4 Palisade Corporation).

Multiple regressions were conducted with the statistical software package SAS with the 'stepwise' selection (significance level p=0.15). The following parameters were included in the analysis: percentage of sand particles (50-2000 μm) [*sand*], years being organically certified [*years*], microbial diversity as determined by DGGE [*H*], species richness as determined by DGGE [*S*], log (nitrate in mg/kg dw) [NO_3], log

(ammonium in mg/kg dw) [NH_4], log (dissolved organic carbon in mg/kg dw) [*DOC*], log (dissolved organic nitrogen in mg/kg dw) [*DON*], log (dissolved organic carbon / dissolved organic nitrogen) [*DOC/DON*], log (C/N) [*C/N*], log (ratio number of copiotrophic bacteria over number of oligotrophic bacteria) [*copio/oligo*], log (biomass carbon in mg/kg dw) [*biomass*], log (moisture content in percentage of total weight) [*moist*], log (dissolved organic carbon per unit biomass carbon x 10) [*DOC/biomC*], log (dissolved organic nitrogen per unit biomass carbon x 10) [*DON/biomC*], and log (basal respiration per unit biomass carbon in mg/kg x 100) [CO_2*/biomC*]. The pH was included after the following normal transformation: arsin(sqrt(pH/pH_{max})) [*pH*]. In order to avoid possible nonlinear relations between the dependent and independent variables because they do not share the same underlying distribution, regression analyses were conducted on normalized data. When variables included in the model were significantly correlated, the variable with the lowest contribution to the model was removed and the regression analysis was repeated to allow other variables to enter the model. Multiple regression was conducted for the whole set of 36 soils and for each management type and soil type separately. Alternative models were assessed by removing the main predictor and running the regression analysis again. Only those alternative models performing equally or better with respect to the amount of variation explained (R^2) were mentioned.

5.3 Results

5.3.1 Model performance.

Although the survival curves were close to (log)linear, they showed a concave curvature (fig. 1A). The Weibull model for survival in all 36 soils had a mean R^2 of 0.98 and a mean RMSE of 0.26 (log CFU) while the linear model showed a mean R^2 of 0.92 and a mean RMSE of 0.36. Residuals obtained from the Weibull model were normally distributed for all fits, displaying no systematic tendencies to be positive or negative. Although the residuals from the linear model were also normally distributed for all fits they deviated more from a normal distribution compared to the residuals of the Weibull model. Since the Weibull model performed best in describing the survival of *E. coli* O157:H7 in manure-amended soil and none of the fits were unsatisfactory, the Weibull model parameters were further analyzed. The observed and modelled values of *E. coli* O157:H7 density over time were highly correlated ($r=0.96$, $p<0.0001$) (fig. 1B). The goodness-of-fit statistics (R^2 and RMSE) did not differ significantly between survival curves in sand compared to loam, ORG versus CONV and between the four combinations, indicating that the Weibull model is suitable to fit survival curves of *E. coli* O157:H7 in an array of different manure-amended soils equally well.

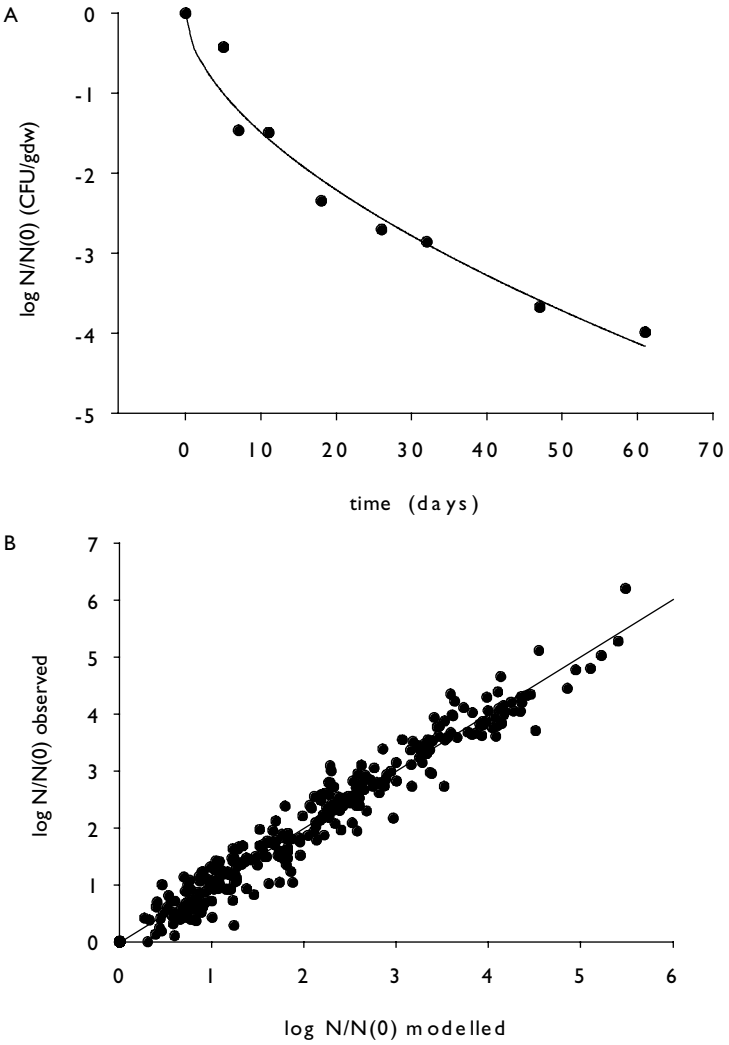

Fig. 1. A) representative example of *E. coli* O157:H7 survival in manure-amended soil showing observed values (♦) and fitted Weibull decline curve (solid line). B) plot of the correlation between observed and modelled values for log N/No for survival in all 36 soils.

The variability in decline kinetics of *E. coli* O157:H7 in 36 different soils were assessed by histograms of the Weibull model parameters and by fitting the data to probability density functions (fig. 2). Based on the Anderson-Darling (A-D) goodness-of-fit statistic, the variability in the Weibull parameters *b* and *n* could be best described by log-logistic distributions, while *ttd* could be best described by a logistic distribution (A-D test statistics respectively 0.325, 0.385 and 0.204; all $p>0.1$).

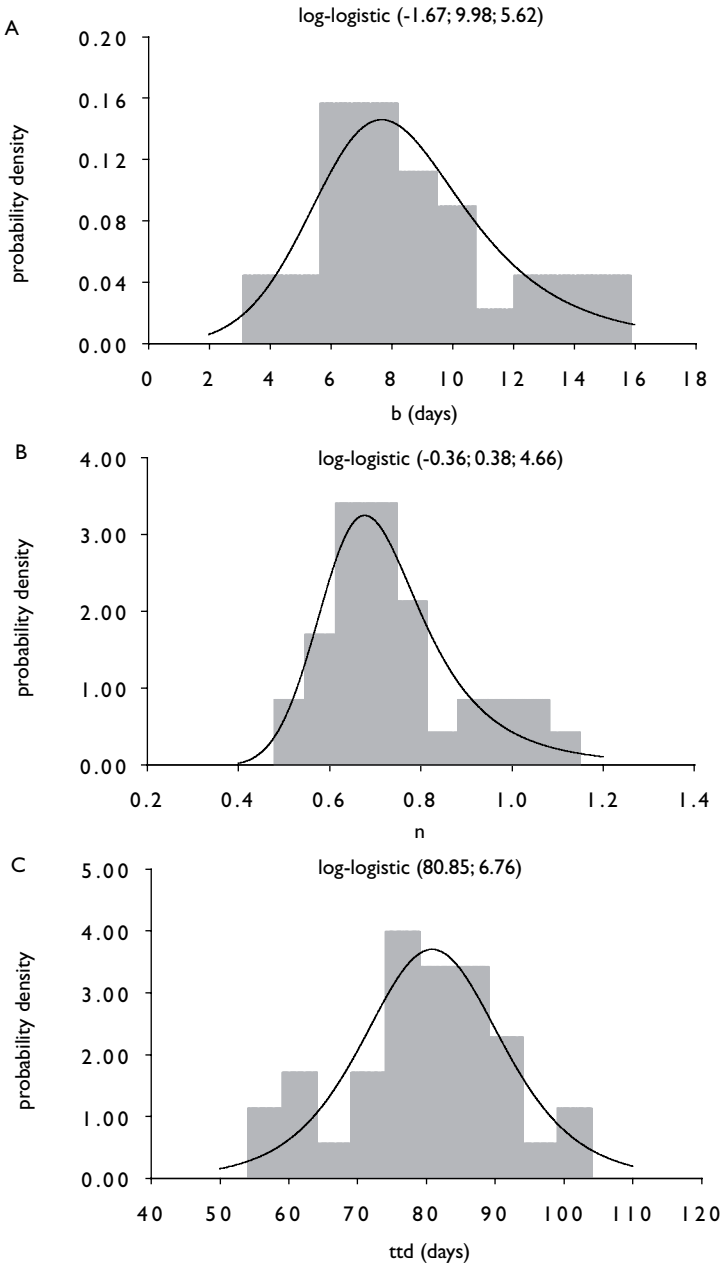

Fig. 2. Histograms and fitted probability distribution function of the Weibull decline curve parameters b (first decimal reduction time) (A), n (shape parameter) (B) and the derived ttd (time to detection limit) (C) for the survival of *E. coli* O157:H7 in 36 different soils.

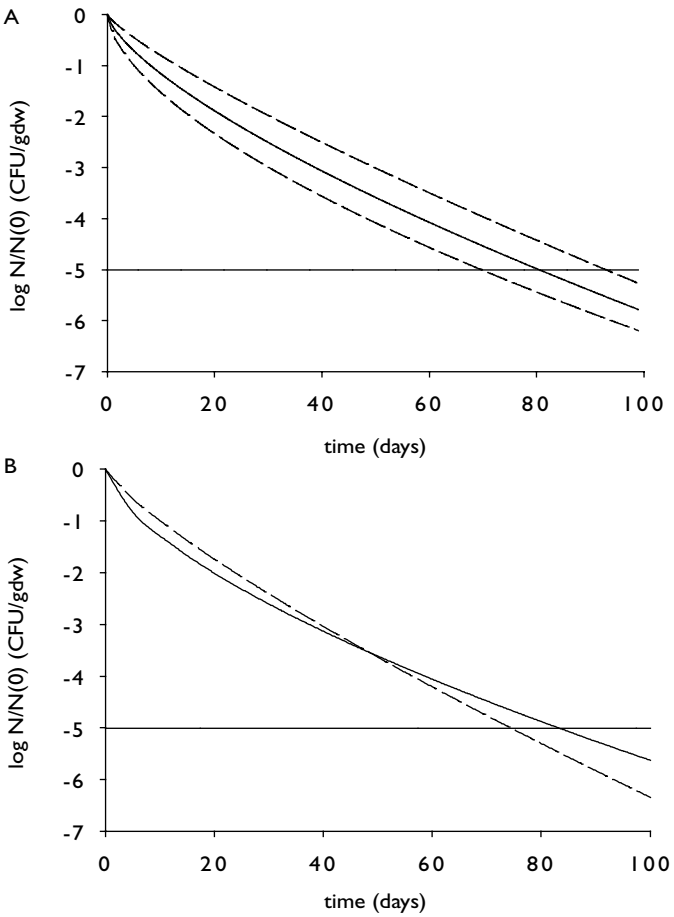

Fig. 3. Average Weibull decline curve of survival of *E. coli* O157:H7 in A) all 36 manure-amended soils (solid) ± 1 standard deviation (dotted) and B) in sand (solid) versus loam (dotted).

5.3.2 Effects of soil type and soil management on survival

The majority of the survival curves showed a concave shape, with a relatively fast initial decline followed by a slower decline phase (correlation between parameters *b* and *n* was 0.91, $p<0.001$). Survival times (determined as the time needed to reach the detection limit *ttd*) of *E. coli* O157:H7 was on average 79.8±12.7 days (S.E. of the mean = 2.11, 95%CI: 75.5 – 84.1, lowest = 54, highest = 105) (fig. 3A). Multivariate analysis of variance revealed that soil type affected the overall decline kinetics significantly ($p=0.006$, Wilks Lambda = 0.658). The first decimal reduction time *b* and the shape parameter *n* were higher for loamy soils (mean b = 10.3 days, mean n = 0.81) compared to sandy soils (mean b = 6.8 days, mean n = 0.64) (both $p<0.0001$) (fig. 4). This

combination of a faster initial decline and a more concave shape of the decline curve for sand compared to loam, resulted in crossing of the average survival curves of both soil types after 54 days (fig. 3B). Although the overall survival, expressed as the time to detection level *ttd*, was longer for sandy soils (mean = 84 days) compared to loamy soils (mean = 78 days), no significant difference in the *ttd* were observed (p=0.151) (fig. 4). The significant effect of soil type on parameters *b* and *n* was present within both ORG and CONV soils. Soil management (ORG versus CONV) did not show an overall effect on the combined variance of the three parameters (p=0.109, Wilks Lambda = 0.815). The first decimal reduction time was significantly lower for ORG sandy soils

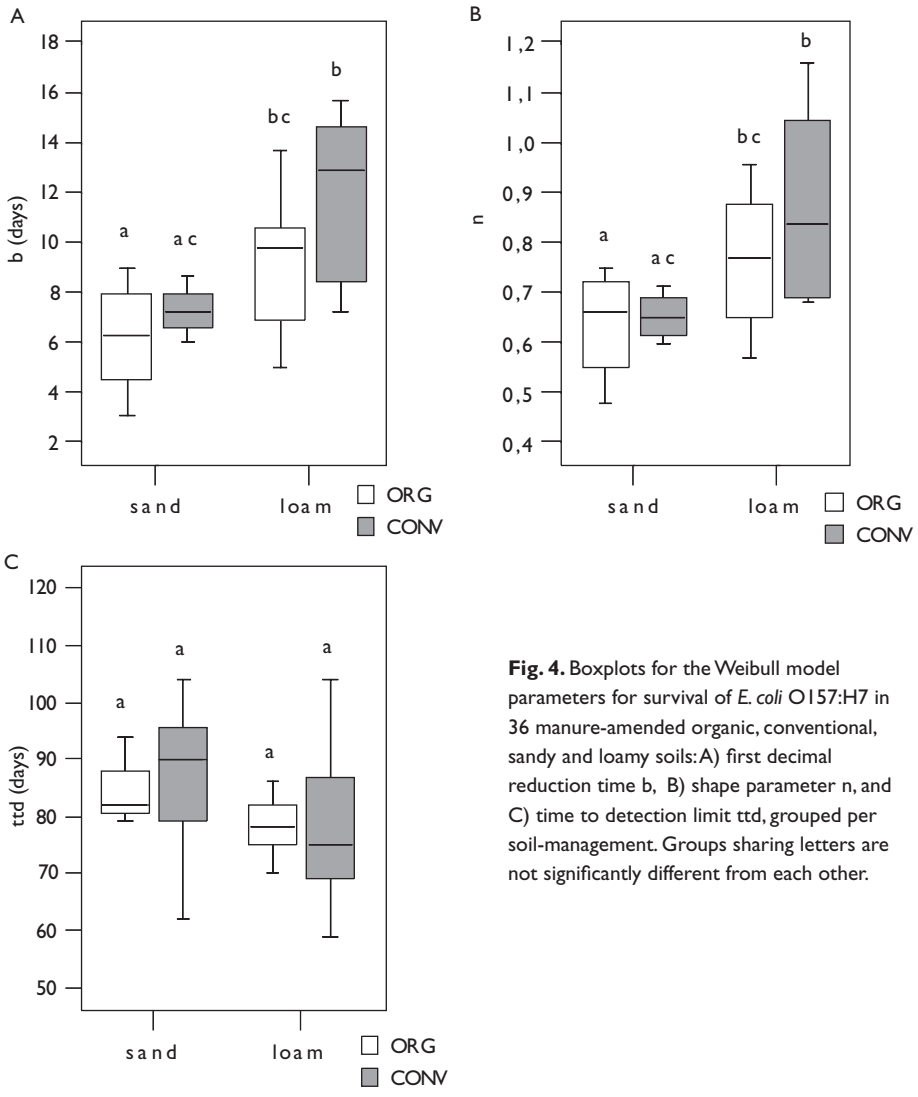

Fig. 4. Boxplots for the Weibull model parameters for survival of *E. coli* O157:H7 in 36 manure-amended organic, conventional, sandy and loamy soils: A) first decimal reduction time b, B) shape parameter n, and C) time to detection limit ttd, grouped per soil-management. Groups sharing letters are not significantly different from each other.

compared to CONV loamy soils ($p<0.0001$) and lower (but just not significant) for ORG loam compared to CONV loam ($p=0.061$) (fig. 4). No effects of management were found on parameter *n* and *ttd*.

5.3.3 Differences in soil characteristics between soil type and management regime.

The *pH* and *copio/oligo* ratio were significantly higher for loamy soils (both $p<0.0001$) while the NH_4 content was higher ($p<0.0001$) for sandy soils, especially in CONV sandy soils. None of the measured soil characteristics differed significantly between ORG and CONV manure-amended soils, although the microbial species richness *S* tended to be higher in ORG sand compared to CONV sand ($p=0.064$). This microbial species richness *S* was positively correlated with the number of years of certified organic management ($r=0.34$, $p=0.045$), indicating a higher species richness with increasing years of organic management.

5.3.4 Relation between survival and manure-amended soil characteristics.

Considering all 36 soils, the time needed to reach the detection limit *ttd* showed a positive correlation with the level of dissolved organic carbon [*DOC*] and the DOC per unit of biomass [*DOC/biomC*] (Table 1, fig. 5). Also within sandy soils the *ttd* was positively correlated with *DOC/biomC*. The *ttd* of loamy soils was positively correlated with *DOC/biomC*, CO_2*/biomC* and negatively with *biomass* and *sand*. Within CONV soils the *ttd* positively correlated with *DOC/biomC* and NO_3 and negatively with *pH*. In ORG soils *ttd* was significantly positively correlated with *DOC* and *DON* but only at the 0.1 significance level with *DOC/biomC*.

Multiple regression analysis revealed a uniform picture with respect to identification of factors most explaining the variation in survival times (Table 2). The *ttd* within all 36 soils as well as within sandy soils, loamy soils (after excluding the percentage of sand particles as a variable) and CONV soils was best explained by the *DOC/biomC*. In all cases the relation between *ttd* and *DOC/biomC* was positive. The remaining variation in *ttd* over all 36 soils was best explained by the ammonium content (NH_4, positive relation) and the number of years under organic management (*years*, negative relation). Within loamy soils the variation in *ttd* was best explained by the physical composition of the soil, i.e. the fraction of sand particles relative to clay particles (negative relation). When excluding *sand*, *DOC/biomC* was also the main determinant of *ttd* within CONV soils. The remaining variation was best explained by *biomass* (negative relation). Within ORG soils *DOC* and *DON* were the best predictors for *ttd* (positive relations). Remaining variation could be explained by microbial species diversity *H* (negative relation).

Table 1. Pearson correlations between the time needed to reach the detection limit (*ttd*) and manure-amended soil characteristics

		sand	*DOC*	*DON*	*biomass*	*DOC/biomC*	*CO_2/biomC*	*NO_3*	*pH*
All samples	ttd		+ 0.33*	+ 0.30		+ 0.52**			
Sand	*ttd*					+ 0.75**			
Loam	*ttd*	- 0.54*			- 0.44*	+ 0.46*	+ 0.45*		
ORG	*ttd*		+ 0.58**	+ 0.63**		+ 0.36			
CONV	*ttd*					+ 0.64**		+ 0.52*	- 0.49*

No value: no relation

No asterisk: correlation at the 0.1 level (2-tailed)

* significant at the 0.05 level (2-tailed)

** significant at the 0.01 level (2-tailed)

Table 2. Best regression models for the time needed to reach the detection limit *ttd* (based on the Weibull decline model) for *E. coli* O157:H7 in 36 manure-amended soils (organic/conventional, sand/loam), based on 16 different abiotic and biotic characteristics of the manure-amended soils.

Group	Model *ttd* =	p-value	R^2
Overall	$46.49^{***}(\pm 8.14) + 20.03^{***}(\pm 4.54) \times$ ***DOC/biomC*** $[R^2_{part}=0.27] + 13.49^{**}(\pm 4.92) \times$ ***NH_4*** $[R^2_{part}=0.13] - 0.56^{*}(\pm 0.34) \times$ ***years*** $[R^2_{part}=0.05]$	<0.001	0.45
Sand	$60.10^{***}(\pm 6.21) + 22.57^{***}(\pm 5.51) \times$ ***DOC/biomC*** $[R^2_{part}=0.57]$	0.001	0.57
Loam	$78.19^{***}(\pm 12.15) - 0.44^{***}(\pm 0.15) \times$ ***sand*** $[R^2_{part}=0.30] + 3.98^{**}(\pm 1.77) + 11.49^{*}(\pm 6.56) \times$ ***DOC/biomC*** $[R^2_{part}=0.16]$	0.005	0.46
	$77.11^{***}(\pm 16.36) + 12.93^{**}(\pm 7.99) \times$ ***DOC/biomC*** $[R^2_{part}=0.21] - 6.53^{*}(\pm 4.26) \times$ ***biomass*** $[R^2_{part}=0.10]$	0.043	0.30
ORG [a]	$47.62^{***}(\pm 19.24) + 43.57^{***}(\pm 6.76) \times$ ***DON*** $[R^2_{part}=0.61] - 20.09^{**}(\pm 9.93) \times$ ***H*** $[R^2_{part}=0.09]$	<0.001	0.69
CONV [b]	$127.65^{***}(\pm 23.50) + 22.72^{***}(\pm 5.51) \times$ ***DOC/biomC*** $[R^2_{part}=0.41] - 57.15^{***}(\pm 18.02) \times$ ***pH*** $[R^2_{part}=0.25]$	0.001	0.66

* $p<0.15$, ** $p<0.10$, *** $p<0.05$.

[a] Organic; alternative model: *ttd* = -9.11 (±14.53) + $48.52^{***}(\pm 8.08) \times$ ***DOC*** $[R^2_{part}=0.60] - 18.86^{**}(\pm 10.41) \times$ ***H*** $[R^2_{part}=0.07]$, $p<0.001$, $R^2=0.67$.

[b] Conveniotnal; alternative model: *ttd* = $126.02^{***}(\pm 27.91) + 14.48^{***}(\pm 5.67) \times$ ***NO_3*** $[R^2_{part}=0.27] - 55.26^{***}(\pm 20.99) \times$ ***pH*** $[R^2_{part}=0.18] + 7.73^{**}(\pm 3.83) \times$ *CO_2/biomC* $[R^2_{part}=0.13]$, $p=0.009$, $R^2=0.58$.

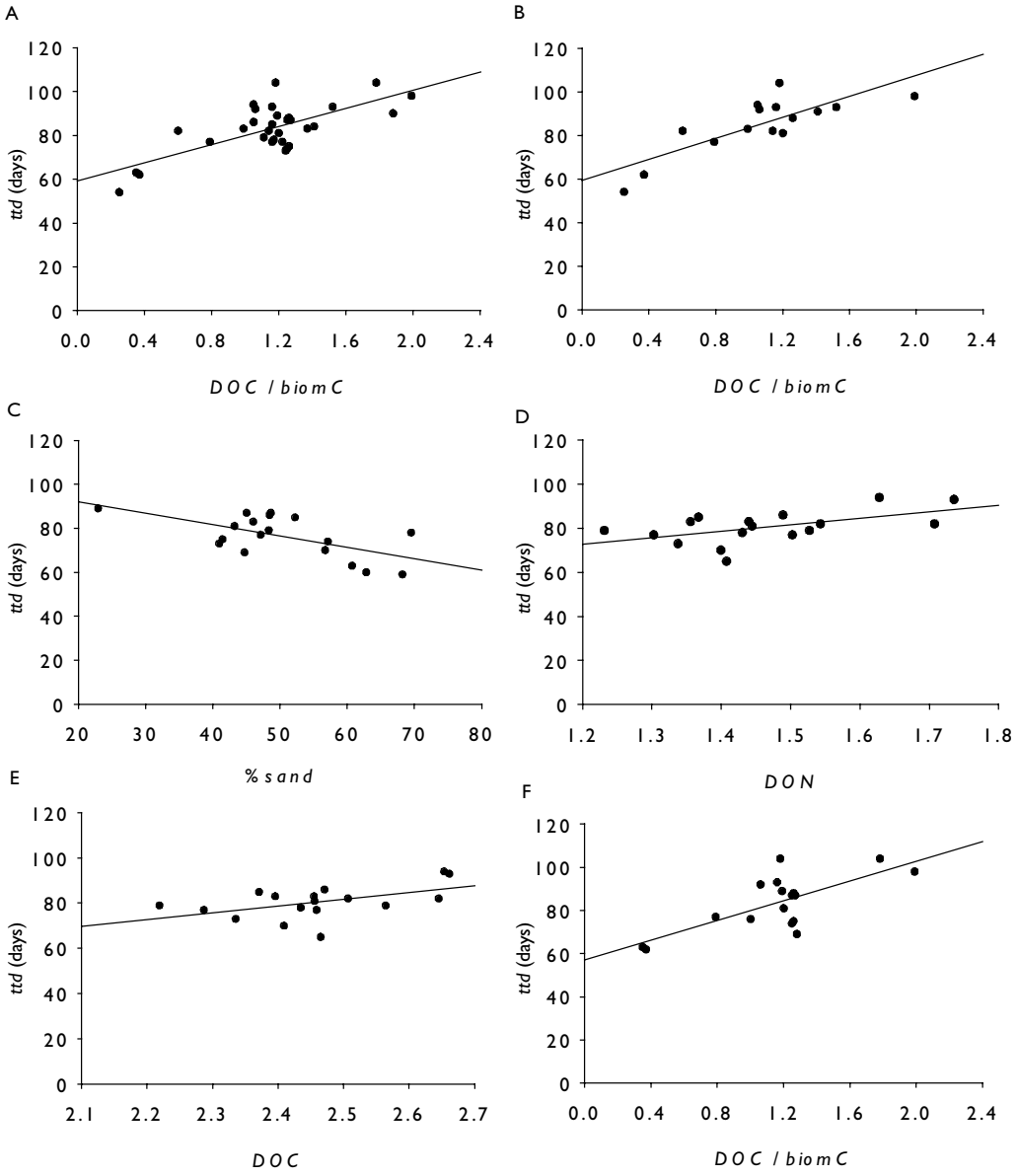

Fig. 5. Scatterplots and correlations between the time needed to reach the detection limit *(ttd)* and A) *DOC/biomC* (unitless log value) in all 36 soils, B) *DOC/biomC* (unitless log value) in the sandy soils, C) sand (%) in the loamy soils, D) *DON* (log mg/kg) in the organic soils, E) *DOC* (log mg/kg) in organic soils and F) *DOC/biomC* (unitless log value) in the conventional soils.

Over all 36 soils the *DOC/biomC* was negatively correlated with the percentage of sand particles (*sand*) and positively correlated with the microbial activity per unit biomass (CO_2*/biomC*). In addition, over all 36 soils as well as within the separate groups (sand, loam, ORG, CONV) the *DOC/biomC* was positively correlated with the moisture content (*moist*).

5.3.5 Extremes and management history.

Although on average no differences in overall survival time of *E. coli* O157:H7 were found between soils with different management and between different soil types, some interesting differences were detected between the four best (*ttd* of 104, 104, 98 and 92 days) and four worst supporting soils (*ttd* of 54, 59, 60 and 62 days). The four most supporting soils were all CONV soils (three sand, one loam) and showed a significantly higher value for shape parameter *n* ($p=0.03$), *ttd* ($p<0.001$), NO_3 ($p=0.048$) and *DOC/biomC* ($p=0.040$). The four worst supporting soils included two ORG and two CONV soils (one sand and loam each).

The four soils with slowest decline of *E. coli* O157:H7 all had a history of slurry and artificial fertilizer application (three only slurry and one slurry with additional artificial fertilizer) while three of the four soils with the fastest decline generally received manure with a higher C/N ratio (two received a mixture of slurry and farmyard manure, one only composted farmyard manure and one only slurry). This relation between pathogen survival and the history of manure type application to the fields was also visible within the whole set of 36 soils. In general the decline of *E. coli* O157:H7 was increasing with increasing history of solid manure with relatively high C/N ratio (fig. 6).

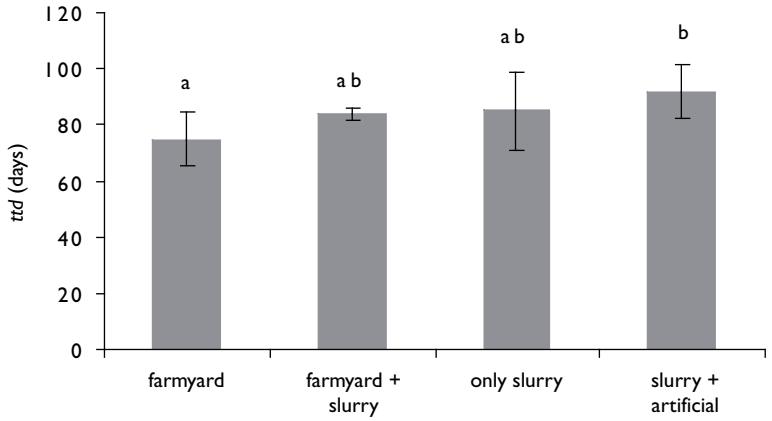

Fig. 6. Survival time of *E. coli* O157:H7 in manure-amended soil per type of manure management history of the original production field. Groups sharing the same number do not differ significantly from each other.

5.4 Discussion

5.4.1 Performance of the Weibull model.

The Weibull model proved to be a suitable model for describing the decline of *E. coli* O157:H7 in manure-amended soil. The model is sufficiently flexible to account for different survival patterns (linear when $n=1$, concave when $n>1$ and convex when $n<1$) and has been previously used to model thermal pathogen deactivation (57) and the survival of soilborne plant pathogens (50). An additional advantage of the Weibull model is the absence of a lower asymptote which can overestimate the survival over prolonged periods (9). With the present study the shape parameter n was different from 1 in all but two cases, which indicates that the use of first-order decline models to describe the survival of *E. coli* O157:H7 in manure-amended soil is not justified. Even though the Weibull model is an empirical model, it can be linked to physiological properties at population level. The model is based on the assumption that the population is heterogeneous with respect to the stress encountered in the substrate. This variability in stress is assumed to follow a Weibull distribution and therefore the survival over time can be modelled with the cumulative form of the distribution. A convex curve ($n > 1$) would mean that the remaining cells become increasingly susceptible to stress, which can be interpreted as evidence that accumulated damage weakens the survivors (and hence their destruction rate will increase with time) (45, 57). A linear semi-logarithmic survival curve ($n = 1$) means that the probability of dying does not depend on time, i.e. there is no effect of accumulated damage and/or there is no biological variation in the pathogen population, antagonistic microbial community or predatory activity. In the present study, the average value of n is smaller than 1 (0.74), which resulted in a concave curve. This can be interpreted as evidence that sensitive members of the population are rapidly eliminated and that the sturdier survivors remain (45). This might be the result of intrinsic biological variation within the population that is already present at the start of the exposure to stress, or of the variation evolving during the exposure time because cells adapt to the encountered stress (57). Alternatively, the antagonistic microbial community or predatory fauna could change over time, resulting in decreasing stress for the surviving cells of *E. coli* O157:H7. Since we obtained different survival curves while using one single inoculum culture and one identical inoculum carrier, it is most likely that changes in (biological) soil characteristics determine whether cells can survive and to what extent and rate. Different soils will react differently to the addition of manure, which results in different nutrient conditions (as expressed by e.g. *DOC/biomC*), microflora and fauna and thereby different survival patterns for *E. coli* O157:H7.

5.4.2 Survival time.

The main objective of this study was to assess the variability in decline kinetics of *E. coli* O157:H7 in a range of different soils and to identify critical factors that determine the rate of decline. Although comparison with other studies is difficult because of the use of different experimental setups and different statistical analyses, the range of survival times (54 to 105 days, average 80) determined in the present study corresponds to those published earlier (6, 38, 40, 64). The determination of *E. coli* O157:H7 survival in 36 soils allowed for the quantification of the variability in overall survival times, which followed a logistic distribution. The shape of this distribution indicates that the chances of a longer and a shorter than average survival are approximately equal. The probability distributions of the model parameters and/or the overall survival times for the survival of *E. coli* O157:H7 in manure-amended soils can be used to increase the accuracy of exposure assessments of vegetables or water contamination.

5.4.3 Difference between soil types and management regimes.

With the present study we found that the values of the log reduction time *b* and the shape parameter *n* of the Weibull model were significantly higher for loamy soils compared to sandy soils. This means that with sandy soils the initial rate of decline of *E. coli* O157:H7 is faster but that the decline rate slows down more with progressing time than with loamy soils. Apparently, *E. coli* O157:H7 is more vulnerable to mortality during the first 1-2 weeks in the sandy manure-amended habitat but survivors are increasingly sturdier compared to survival in the loamy soils. As a result, average survival curves of sandy and loamy soils crossed and no difference was observed in overall survival time between sandy and loamy soils. However, within loamy soils the survival of *E. coli* O157:H7 was primarily determined by the soil texture, with longer survival associated with relatively more clay particles compared to sand particles. Survival of *E. coli* O157:H7 has been reported to be prolonged in finer textured soils (13, 40, 43). However, most published data on the survival of *E. coli* O157:H7 in soil typically included only a limited number of different soils, which does not fully justify generalized conclusions on the effect of soil type. In contrast, the present study included 36 different soils and showed a faster initial decline of *E. coli* O157:H7 in sandy soils but no differences in the final survival time between both soil types.

In general it is stated that finer textured (clayey) soils result in prolonged survival of introduced bacteria compared to coarser textured (sandy) soils because of higher availability of protective pore spaces against feeding by soil fauna like protozoa (61). This could explain the faster initial decrease in *E. coli* O157:H7 numbers in the sandy soils compared to the loamy soils. But for reasons which are unclear at the moment the decline rate in sandy soils decreases with time relative to the decline in loamy soils. A possible explanation might

be that the presence of manure decreased the attachment of fecal coliforms to clay and silt fractions to a higher extent than with sand fractions (19). In addition, with small pore spaces soil moisture and dissolved organic matter are more difficult accessible for microbes (36). Higher clay content was associated with a more negative matric potential (and thus higher water stress) and a faster decline of *E. coli* (40). In the present study we attempted to minimize the possible overruling effect of soil moisture availability by adjusting the soil moisture content to 60% of the water holding capacity. The gravimetric water content did not differ between both soil types during the experiment but the matric potential was not measured. However, survival of *Pseudomonas fluorescence* and *E. coli* O157:H7 was found to be unaffected by the matric potential (39, 47).

No differences in survival of *E. coli* O157:H7 were observed between the soils of both management regimes. This is probably related to the absence of differences in chemical and biological soil characteristics between both management regimes. In contrast, earlier data on 12 soil pairs which were also included in the present study showed significantly higher levels of NO_3, basal respiration rate, numbers of copiotrophic and oligotrophic bacteria, microbial species richness (not the diversity) and nematode diversity in the organically managed soils (60). In the present study, microbial species richness and diversity were higher in organic soils but these differences were not significant. This might be the result of the addition of the same manure as inoculum carrier to all soils and the disturbance of the soil microbial community by sampling and mixing.

5.4.4 Influence of chemical and biological soil characteristics

The single soil property that appeared to be the best predictor of *E. coli* O157:H7 survival in manure-amended soil is the level of dissolved organic carbon per unit of biomass carbon (*DOC/biomC*), i.e. the level of easily available carbon sources per unit of soil biomass. Additional positive relations were found between survival and levels of *DOC*, *DON* and CO_2*/biomC*. When *E. coli* leaves its primary habitat, the nutrient-rich (copiotrophic) anaerobic intestine of warm-blooded animals with an ample supply of carbonaceous compounds (34), it has to adapt to its nutrient-deficient (oligotrophic) and largely aerobic secondary habitat like soil where the concentration of energy sources is usually very low (20). Although *E. coli* can potentially exhibit oligotrophic kinetic properties in chemostat cultures (30), a major factor in *E. coli* die-out in soils is thought to be its inability to lower its metabolic rate to meet the low availability of usable organic carbon and to adjust to conditions of low nutrient availability (24, 29). With the present study we showed that the survival of the copiotrophic *E. coli* O157:H7 in manure-amended soil is indeed longer in more copiotrophic (more *DOC, DON, DOC/biomC* and CO_2*/biomC*) manure-amended soil systems. In addition, increased levels of easily

available energy sources in DOC may (temporarily) decrease the competitive pressure between organisms and thus possibly allow increased persistence

Within ORG soils survival of *E. coli* O157:H7 was predominantly determined by the absolute levels of dissolved organic carbon and nitrogen (*DOC* and *DON*). Although positively related, the *ttd* was not correlated with the *DOC/biomC*, like in CONV sandy, and loamy soils. The absence of *DOC/biomC* as an explanatory factor for the variability in *ttd* in the regression model for ORG soils is probably the result of the lower variability in *biomass* between the ORG soils compared to the CONV soils (standard deviation from mean respectively 0.49 and 0.66). In addition to the effects of *DOC* and *DON*, a negative correlation was found between *ttd* and the microbial diversity H (as measured by DGGE) with a lower *ttd* with increasing *H*. Soils or rhizospheres with higher microbial diversity are expected to be more resistant to stress and disturbances and consequently less susceptible to invasion (28, 33, 37). The results of the present study indicate a potential importance of an increased microbial diversity in managing the survival of human pathogens.

Within CONV soils the survival time of *E. coli* O157:H7 was negatively related to the pH of the manure-amended soil. In contrast, several studies indicated a shorter survival of enteric bacteria in soils with lower pH (17, 52). However, it must be stressed that these studies all considered the survival of a generic *E. coli* strain and not a pathogenic strain like *E. coli* O157:H7. When grown in broth, pathogenic *E. coli* are significantly more acid tolerant than non-pathogenic strains (18). The virulence of *E. coli* O157:H7 is thought to be at least partially dependent on its ability to survive the low pH of the gastric stomach (32). Therefore, it is likely that *E. coli* O157:H7 has some selective advantage over other bacteria at lower pH. This could also explain the decreasing decline rate with progressing time with the more acidic sandy soils compared to loamy soils.

5.4.5 Management factors to control the survival of *E. coli* O157:H7

Although no overall difference was found in *E. coli* O157:H7 survival in organic and conventional manure-amended soils, certain soil management aspects can be crucial to the control of human pathogens in soil systems. It should be realized that although we added the same amount of identical manure to each of the 36 soils, we found a considerable range within all measured chemical and biological variables in manure-amended soils. This means that levels of the measured variables depend on intrinsic soil characteristic and how the soil system reacts to the addition of manure.

The results of the present study indicate that the survival of *E. coli* O157:H7 is less prolonged under more oligotrophic conditions (i.e. lower levels of *DOC, DON* and *DOC/biomC*). Oligotrophication of agricultural ecosystems, which means the reduction of mineral nitrogen, soluble carbon compounds and available phosphorus, is thought to

increase the natural suppression of plant diseases (59). Such a soil system can be achieved by the regular addition of organic fertilizers characterized by a relatively high C/N ratio, like solid animal manure and compost of plant or animal origin to the soil (58). Indeed, *E. coli* decreased more rapidly in soils treated with solid beef cattle manure compared to soils treated with liquid swine manure (56). In addition, soils with a high content of readily available nutrients showed a positive selection for α- and γ-proteobacteria, being indicative of r-selection, which is selection for bacteria with potentially high growth rates like *E. coli* O157:H7 (53). In low-nutrient soil or soil with a high content of recalcitrant substrates, the percentage of *Acidobacterium* increased, being indicative of k-selection (selection for bacteria with lower growth potential but higher capability to compete for substrates).

Improvement of manure quality receives increased attention in order to reduce ammonia emission, nitrate leaching, stimulate microbial activity in the soil and increase soil organic nitrogen content (46). This can be achieved by increasing the C/N ratio of the manure, which in turn can be achieved by feeding cattle a diet with a higher fibre content or mixing manure with straw. In addition, *E. coli* O157:H7 has been found to decline significantly faster in manure derived from cattle which were fed high fibre diet compared to a low fibre diet (14). When applied to soil, this results in a relatively slow release of easily available nitrogen and carbon sources and increased net nitrogen immobilization in the soil. This creates a more oligotrophic system which is a disadvantage for the copiotrophic *E. coli* O157:H7. Fertilizer of low quality (like slurry or artificial fertilizer) would have the opposite effect since this is relatively rich in readily available carbon and nitrogen sources, which in turn can lead to eutrophication of the soil. The application of liquid manure to soil was found to bring triple the amount of water-extractable organic carbon compared to the application of solid manure (2). Moreover, urea-based and ammonium-based fertilizers temporarily solubilise soil organic matter and can induce a marked increase in dissolved organic carbon content (8). An eutrophicated soil system will not only be subjected to higher nutrient losses (46) and lower natural suppression of plant pathogens (58) but also to an increased survival of human pathogens like *E. coli* O157:H7, which implies an increased risk of spread and transfer of this pathogen into the food chain.

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Chapter 6

Quantitative exposure assessment for the contamination of lettuce with *Escherichia coli* O157:H7 from manure-amended soil.

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Abstract

The objective of the present paper was the development of a quantitative microbial exposure assessment model for the contamination of lettuce with *E. coli* O157:H7 from manure-amended soil under constant environmental conditions. As much as possible Dutch data was used to reflect the Dutch primary production chain of lettuce. The transmission of *E. coli* O157:H7 was modeled probabilistically through the primary production chain of lettuce, taking the pathogen prevalence and densities in different ecological habitats into account (cattle, manure, manure-amended soil and lettuce). The baseline model estimated an average presence of 0.34 (5^{th} percentile: 0.03, 95^{th} percentile 0.92) contaminated lettuce plants per hectare (on average 1 in $3.27 \cdot 10^5$ produced heads in the Netherlands). Sensitivity analysis revealed that the likelihood of exposure was most sensitive to the prevalence of contaminated manure, the manure storage time and the initial density of *E. coli* O157:H7 in naturally contaminated manure. Testing these hypothetical intervention strategies with realistic data revealed that the incorporation of a minimum manure storage time of 30 days and a minimum fertilization-to-planting interval of 60 days, were most successful in reducing the number of contaminated lettuce heads. A traditional organic production scenario resulted in a 71% reduction in the expected number of contaminated lettuce plants (0.10 contaminated plants per hectare, 5^{th} percentile: 0.01, 95^{th} percentile: 0.24), while an intensive conventional scenario resulted in an increase of 62% (0.89 contaminated plants per hectare, 5^{th} percentile: 0.06, 95^{th} percentile: 2.42). In order to perform a full scale risk assessment, knowledge gaps need to filled (pathogen dynamics in and on plants during growth) and the processing of lettuce (slicing, washing and packaging) should be included.

6.1 Introduction

During recent years a growing number food-borne illnesses has been associated with the consumption of fresh produce (59, 65). Produce was responsible for 22% of all foodborne disease outbreaks in the US between 1990 and 2004, which makes this the second most implicated single-food vehicle after seafood (33% of outbreaks) and before poultry (18%), beef (16%) and eggs (13%) (4). Interestingly, produce accounted for the highest proportion of disease cases (38%), while seafood accounted for the lowest proportion (12%) (4). Produce also showed the highest number of cases per outbreak (on average 49 compared to 10, 30, 28, and 32 cases for respectively seafood, poultry, beef and eggs. In England and Wales fresh produce accounted for 5.5% of the total number of foodborne disease outbreaks between 1992 and 2000 (42).

From 1973 to 1997, lettuce was the main produce item responsible for single produce-associated outbreaks in the United States (25 out of 85 outbreaks) (59). *E. coli* O157:H7 and lettuce were found to be strongly associated with each other: 29% (5 out of 17) of all lettuce-related outbreaks were due to *E. coli* O157:H7 and 38% (5 out of 13) of all *E. coli* O157:H7 outbreaks with fresh produce were associated with lettuce. Recently (November-December 2006), a multi-state *E. coli* O157:H7 outbreak occurred as a result of the consumption of shredded iceberg lettuce as an ingredient in a specific fast-food restaurant, resulting in 61 confirmed cases, of which 53 (75%) were hospitalized and 8 (11%) developed HUS. In Europe, 18 microbial alert-notifications for lettuce (10) and spinach (8) have been described by the Rapid Alert system of the European Union from 2004 to 2007 (http://ec.europa.eu/food/food/rapidalert/index_en.htm). Several outbreaks of *E. coli* O157:H7 in the US and Europe associated with lettuce have been described in detail (2, 32, 60, 74).

Produce can become contaminated at any point during the primary production, processing and distribution chain. For pathogens with animal reservoirs, like STEC *E. coli* O157:H7, contamination in the field can occur when contaminated manure is used for fertilization. *E. coli* O157:H7 bacteria can become epiphytically associated with the surface of vegetables grown in contaminated manure-amended soil (35, 45), where they subsequently can survive and even grow (1). Recently it has been demonstrated that *E. coli* O157:H7 can colonize the leaf tissue of lettuce also endophytically during growth in contaminated manure-amended soil (22, 61), which may constitute a public health risk since these bacteria are unlikely to be removed during post-harvest sanitation or washing by consumers. The endophytic presence in lettuce leaf tissue has also been demonstrated for *Salmonella* Dublin (38).

Prevention of pre-harvest contamination of fresh produce is an essential part of a systems approach focused on developing interventions designed to achieve delivery of microbiologically safe produce to consumers. Cattle constitute a reservoir for *E. coli*

O157:H7, and cattle manure is frequently the main vehicle for the spread of this pathogen to vegetables. Because animal manure is a primary source for fertilization in organic farming, microbial safety is at the centre of attention for organic vegetable production. However, it has not been demonstrated that the risk of contamination would be higher with organic compared to conventional production. In Europe (and the Netherlands in particular) cattle manure is used as the primary source for fertilization in organic as well as in conventional vegetable production.

Suppression of human pathogens in manure, manure-amended soil and the subsequent prevention of spread into the food chain by contamination of produce must be realized by intrinsic factors determining survival in the various stages of the production chain, since anti-bacterial pesticides are not available for the primary production chain. Recently it was demonstrated that survival of *E. coli* O157:H7 in cattle manure is significantly longer when the animals are fed a low fibre / high energy diet (grass/maize silage) compared to a high fibre diet (straw) (21). In addition, higher levels of easily available substrates in soil were found to result in prolonged survival of *E. coli* O157:H7 (20)

It is virtually impossible to allay or justify the concerns with respect to the potential preharvest contamination of vegetables through traditional hypothesis testing. Infection rates are expected to be so low that sample sizes needed for adequate statistical power make such studies impracticable. A more pragmatic approach is the application of quantitative microbial risk assessments (QMRA), which is a powerful tool to estimate order-of-magnitude risks associated with certain scenarios. Probabilistic QMRA modelling is increasingly used as a tool to evaluate food related health risks, like for *E. coli* O157:H7 in ground beef (13, 16), *Salmonella* in pasteurized liquid eggs (75), *Listeria* in trout (41) and soft cheese (9) and *Campylobacter* in poultry meat (67). Several risk assessment studies have been performed for the contamination of vegetables with viruses by reclaimed irrigation water (6, 28, 50, 58, 64, 69). The number of human infections in the UK through consumption of root crops grown on agricultural land to which sewage sludge has been applied was quantified for seven pathogens, including *E. coli* O157:H7 (24, 25). The approach adopted was to use arithmetic mean values in an event tree to calculate the arithmetic mean exposure value, which ignores variation and was considered worst-case.

Some attention has been paid to estimating health risks associated with the consumption of leafy vegetables grown in soil to which sewage sludge is applied (37, 73). However, no exposure or risk assessment models are available for the pre-harvest contamination of leafy vegetables grown in soil enriched with manure. The objective of the present paper was the development of a probabilistic quantitative microbial exposure assessment model for the contamination of lettuce with *E. coli* O157:H7 from manure-amended soil under constant environmental conditions. The factors contributing most to the final exposure were identified and the effects of various risk mitigation strategies were assessed.

6.2 Materials and methods

6.2.1 Model description

The exposure model was designed to estimate the likelihood of contaminated lettuce plants at the moment of harvest and the densities of pathogenic cells likely to be present. The model only considered contamination of lettuce with *E. coli* O157:H7 by the application of contaminated dairy manure; the possible contamination of lettuce plants by other pathways like wild-life or irrigation was not considered. The transmission of *E. coli* O157:H7 was modeled through the primary production chain of lettuce, from its reservoir (dairy farms) to the moment of harvest. The transmission model followed probability distributions of the prevalence and concentration of the hazard along several separate modules, reflecting the different ecological habitats that the pathogen passes during its spread from dairy cattle to lettuce crops (cattle, manure, manure-amended soil and lettuce). A schematic diagram of the model is shown in Fig. 1. The model was constructed using Microsoft Excel with @ Risk add-in software (version 4.5.5 2004, Palisade Corporation, Newfield, NY, USA). A

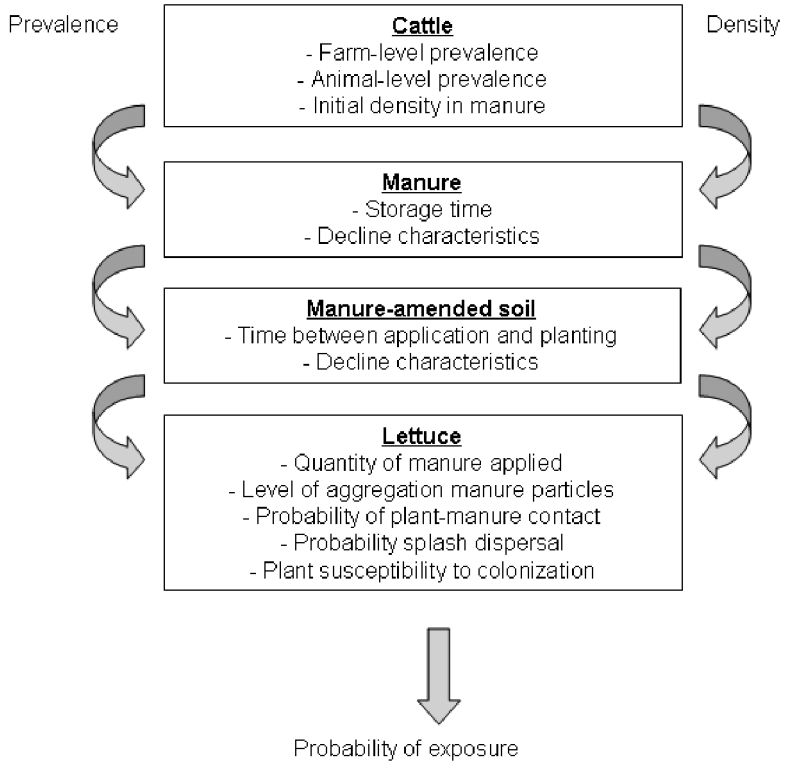

Fig. 1. Schematic overview of the exposure model.

Monte Carlo simulation of the model sampling was performed with Latin Hypercube (10 000 iterations) using probability distributions describing the variability and uncertainty of the input parameters. Input data for the exposure model were obtained from experiments performed within our research group, literature data and expert opinions. The input distributions were based as much as possible on Dutch data. Where no Dutch data were available, international literature data were used. First, a baseline model was created which included all relevant data to obtain a general exposure estimate. Subsequently, the influence of specific organic versus conventional farming practices or specific intervention strategies on the contamination risks were assessed.

6.2.2 Input data and distributions of the baseline model

Prevalence of contaminated dairy manure.

We estimated the prevalence of contaminated manure by the prevalence of *E. coli* O157 positive dairy herds and animals. Several studies reported herd-level prevalence data of *E. coli* O157:H7 for Dutch dairy herds, which are summarized in Table 2. These measured prevalence data depend on the season of sampling, the detection method used, the sensitivity of the method and the way of processing the samples. A beta distribution was considered for herd-level prevalence (*HP*), based on survey data listed in Table 1. Since not all manure from a positive dairy farm is contaminated we used the overall animal-level prevalence (fraction of positive individual animals relative to the total number of animals on a farm) as an estimate for the prevalence of contaminated manure. The animal-level prevalence in positive tested farms (AP^+) was modeled using a uniform distribution with values between minimum 0.8% and maximum 61% due to seasonal effects and test sensitivity of the test used (31, 56). The animal-level prevalence for the negative-tested herds (AP^-) was included as a constant with a value of 0.42% (31), reflecting the prevalence of false-negative herds. The overall animal-level prevalence (*P*) was calculated as $P = HP \cdot AP^+ + (1 - HP) \cdot AP^-$ (46, 71). Seasonality and geographical effects were not incorporated in the model.

Initial *E. coli* O157:H7 concentration in dairy manure.

There are no data available from the Netherlands on the concentration of *E. coli* O157:H7 in cattle manure. Excluding data on experimentally infected cattle, seven literature sources were used to construct a histogram, representing the variability in the concentration of *E. coli* O157:H7 found in manure from naturally colonized cattle (Table 2). This histogram was converted to an empirical cumulative frequency distribution for the initial concentration of *E. coli* O157:H7 in manure (D_{man1}) (13, 70), with a minimum of 0 and a maximum of 8 $\log_{10}$ CFU/g (fig. 2).

Table 1. Description and distributions of input variables and calculations for the base-line model.

Variable	Description	Distribution / model	Values	Source
D_{man1}	Initial density in manure (log CFU/g fresh weight)	Cumulative (min; max; $\{x_i\}$; $\{p_i\}$)	Min: 0; max: 8; $\{x_i$: 1,2,3,4,5,6,7,8$\}$; $\{p_i$: 0.102,0.514,0.650,0.811,0.938,0.994, 0.997,1$\}$)	Table 2
HP	Herd prevalence (fraction of positive herds)	Beta(α; β)	α: 174; β: 1997	Table 3
AP^+	Animal prevalence on positive herds (fraction of individual cows)	Uniform(min; max)	Min: 0.8%; max: 61%	(31, 56)
AP^-	Animal prevalence on negative farms (fraction of individual cows)	Constant	0.42%	(31)
P_{pm}	Overall animal-level prevalence (=prevalence contaminated manure)	$HP \cdot AP^+ + (1-HP) \cdot AP^-$		(46)
D_{man2}	Density in manure after storage (log CFU/g manure dry weight)	$-((T_{man} / d_{man})^{n1}$		
d_{man}	Decimal reduction time of Weibull model for decline in manure (days)	Weibull(α; β)	α: 1.5027; β:18.88; shift(1.6873)	(19, 21)
$n1$	Shape parameter of Weibull model for decline in manure	$0.515 + (0.022 \cdot d_{man}) + e_{man}$		(19, 21)
e_{man}	Residuals relation d_{man} and $n1$	Normal(μ;σ)	μ: -0.0007, σ: 0.09	
T_{man}	Manure storage time (days)	Uniform(min; max)	Min: 1; max: 120	Expert opinion
D_{soil}	Density in manure in soil at planting (log CFU/g dry weight manure)	$-((T_{soil} / d_{soil})^{n2}$		
$n2$	Shape parameter of Weibull model for decline in manure in soil	$(0.044 \cdot d_{soil}) + 0.3524 + e_{soil}$		
d_{soil}	Decimal reduction time of Weibull model for decline in manure in soil (days)	Log-logistic(γ; β; α)	γ: -1.67; β: 9.98; α: 5.62	(Franz 2007)
e_{soil}	Residuals relation d_{soil} and $n2$	Logistic(α; β)	α: 0.0031, β: 0.031	
T_{soil}	Time interval manure application – planting (days)	PERT(min; most likely; max)	Min: 14, most likely, 41, max; 150	Expert opinion
M_{man}	Quantity of manure applied to field (kg manure / ha)	PERT(min; most likely; max)	Min: 10000, most likely: 23000, max: 35000	Expert opinion
C_{man}	Concentration manure particles (kg fresh manue / kg fresh soil)	$1.5 \times 10^6 / M_{man}$	Based on 1ha, 10 cm deep, bulk density 1.5 g cm^{-3}	
P_c	Probability root colliding with manure (per plant root-system)	$1-[1/((C_{man} + k) / k)^k)]$		
k	Aggregation parameter of negative binomial distribution	Uniform(min; max)	Min: 0.001; max: 0.1	
$P(D_{soil}>2)$	Probability $D_{soil} > 2$ (log CFU/g dry weight manure)	Depending on distribution D_{soil}		
P_{con}	Probability of plant contamination from soil (per plant)	Beta(α; β)	α: 13; β: 1	(22)
P_{con1}	Probability of internal contamination from soil (per plant)	Beta(α; β)	α: 4; β: 10	(22)
P_{exp1}	Probability of exposure from soil uptake (per plant)	$P_{pm} \cdot P(D_{soil}>2) \cdot P_c \cdot P_{con}$		
N_{rain}	Number of rain events (#/year)	Binomial(n; P_{rain})		
n	Number of growing days (days)	Round(Uniform(min; max))	Min: 50; max: 75	
P_{rain}	Probability of rain (per day growing season)	Beta(α; β)	α: 201; β: 166	
P_{exp2}	Probability of exposure through splash dispersal (per plant)	$P_{pm} \cdot P(D_{soil}>2) \cdot P_c \cdot N_{rain} \cdot 0.01$		
P_{exp}	Total probability of exposure (per plant)	$P_{exp1} + P_{exp2}$		

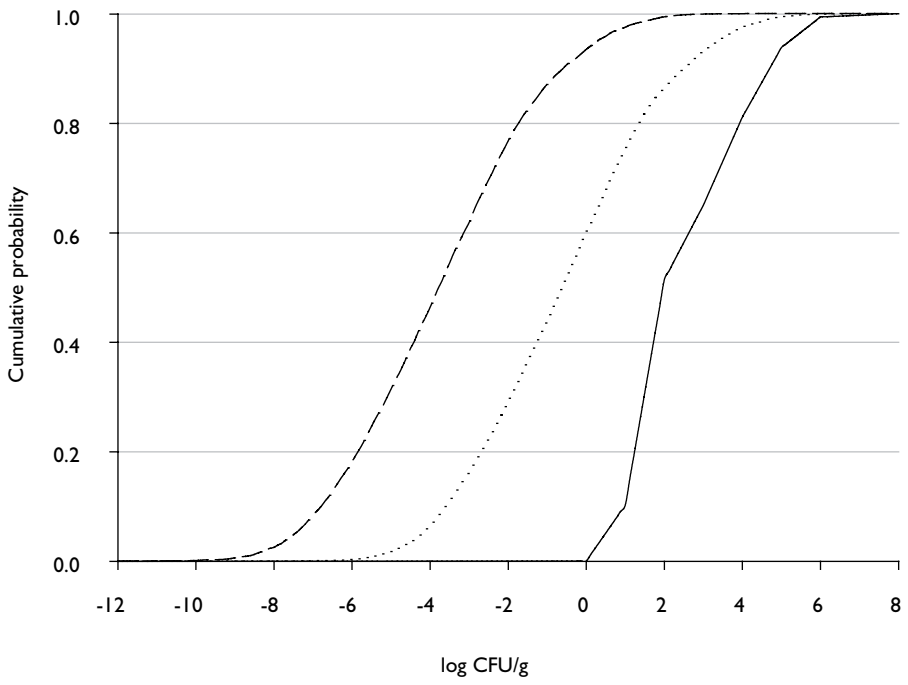

Fig. 2. Cumulative probability curves for the density of *E. coli* O157:H7 in fresh manure (solid line), in manure at the time of applying to the field (dotted line) and in manure particles in soil at the time of planting lettuce seedlings (striped line), as predicted by the exposure model.

Decline in manure during storage.

The variability in decline of *E. coli* O157:H7 densities over time during manure storage was assessed using the survival data from laboratory experiments at 10°C (19, 21). These manures were collected as fresh manure from the stable floor. The survival data from 41 different manures was fitted to the Weibull decline model by nonlinear regression (Gauss-Newton method) (SAS version 8.02, SAS Institute Inc., Cary, USA, 2001): $D_{man2}(T_{man}) = -(T_{man} / d_{man})^{n1}$, where $D_{man2}(T_{man})$ is the relative population size (log N/N_0 in log CFU) at time T_{man} (days), d_{man} (scale parameter) represents the *time of first decimal reduction* (days) and *n1* is the shape parameter. For $n1>1$ a convex curve is obtained, while for $n1<1$ a concave curve is obtained. All fits were significant ($p<0.001$) with an average pseudo-R^2 of 0.95 ± 0.03. The relation between parameters d_{man} and *n1* was best described by a linear function: $n1 = 0.515+(0.022\ d_{man})$ ($R^2=0.95$, $p<0.001$). The envelop method, whereby the value of the independent variable statistically determines the value of the dependent variable, was used to model the dependency between both parameters in a probabilistic way (70). The residuals of the linear relation between both parameters were represented by a normal distribution, which was added to the dependency equation as an error term.

Table 2. Reported densities of *E. coli* O157:H7 in fresh feces of natural contaminated dairy cows.

	Density classes in $\log_{10}$ CFU/g									
Feeding regime	<1	<2	<3	<4	<5	<6	<7	<8	Total	Source
n.r.[1]	0	15	0	2	11	3	0	0	31	(77)
Grain-fed	1	6	3	1	1	0	0	0	12	(17)
Pasture-fed	6	3	3	0	1	0	0	0	13	(17)
Lot-fed	13	6	2	1	0	1	0	0	23	(18)
Grass-fed	13	1	1	1	0	0	0	0	16	(18)
n.r.	0	27	6	7	2	2	0	0	44	(49)
n.r.	0	4	0	0	1	2	0	1	8	(23)
n.r.	0	71	13	10	5	2	0	0	101	(48)
n.r.	0	0	16	30	20	8	1	0	75	(54)[2]
SUM	33	133	44	52	41	18	18	1	323	

[1] Not reported

[2] Counted number of individual samples from fig. 1

This way, for each sampled value of d_{man} a probabilistic estimate of *n1* could be calculated. Finally, the density of *E. coli* O157:H7 cells after manure storage was calculated with the Weibull decline model whereby d_{man} was substituted by a Weibull distribution describing its variability (truncated on the minimum and maximum observed values within the experimental dataset), and *n1* by the probabilistic dependency function. The duration of the manure storage T_{man} was represented by a uniform distribution with all possible storage times between 1 and 120 days having the same probability. Based on literature data, no inoculum-dependent decline kinetics were assumed (8).

Decline in manure-amended soil.

The output concentration of the manure module served as the input for the soil module. The fate of *E. coli* O157:H7 cells in manure-amended soil was modeled per unit manure since it is most likely that cells, which were introduced into the soil by manure, mostly would stay attached to manure particles instead of a homogenous distribution throughout the manure-amended soil mass (except perhaps after a rain storm). Survival data from an experiment including 36 different Dutch soils (20) were recalculated and expressed per g of manure. These data were fitted to the Weibull model as mentioned above for survival in manure: $D_{soil}(T_{soil}) = -(T_{soil} / d_{soil})^{n2}$, where $D_{soil}(T_{soil})$ is the relative population size (log N/N_0 in log CFU) at time T_{soil} (days), d_{soil} represents the *time of first decimal reduction* (days) and *n2* is the shape parameter. All fits were significant ($p<0.001$) with an average pseudo-R^2 of 0.98. Again, the envelop method was used to model the dependency between both model parameters in a probabilistic way. The relation between both parameters was linear: $n2 = (0.0438 \cdot d_{soil}) + 0.358$ ($p<0.001$, $R^2 = 0.83$). The residuals of this relation were best described by a logistic distribution, which was added to the dependency equation as an

error term. The density of *E. coli* O157:H7 numbers in manure-amended soil at time of planting lettuce seedlings (T_{soil}) was calculated with the Weibull decline model whereby d_{soil} was substituted by a log-logistic distribution describing its variability (truncated on the minimum and maximum observed values within the experimental dataset) and *n2* by the probabilistic dependency function. The parameter T_{soil} was represented by a Pert-distribution based on a survey conducted among 28 lettuce farmers throughout the Netherlands.

Plant infection.

Colonization of lettuce by human pathogens like *E. coli* O157:H7 from manure-amended soil was assumed to follow similar patterns as that by soil-borne bacterial plant pathogens. The attachment to the root surface, internalization into the root tissue and spread to the edible part of the lettuce plant have all been demonstrated (22, 61, 72). The attachment to the root primarily depends on the probability that a root collides with a manure-particle containing *E. coli* O157:H7. A negative binomial distribution was used to estimate this probability (P_c) since it is likely that the manure particles are distributed throughout the soil in an aggregated way. The negative binomial distribution was used as described by Wood *et al.* (76). The concentration of manure particles C_{man} was calculated as the amount of manure in 1.5×10^6 kg of soil (based on 1 ha, 10 cm deep incorporation and a bulk density of 1.5 g cm^{-3}). Because of the lack of experimental observations, the value of the aggregation parameter *k* (lower when more aggregated) was estimated by plotting P_c against C_{man} with different values for *k*. For the baseline model a uniform distribution with *k* values between 0.01 and 0.1 was chosen, to represent different kinds of manure and application methods. It is not known what the inoculum threshold of *E. coli* O157:H7 is in order to successfully colonize the roots and the edible plant parts. *E. coli* O157:H7 has been shown to colonize the roots of lettuce seedlings and to multiply to higher densities (72). For the plant pathogenic bacterium *Ralstonia solanacearum* it is thought that a minimal amount of approximately 1000 cells per gram of soil is required to cause successful root colonization (Van Overbeek 2007, *personal communication*). Since *E. coli* O157:H7 can be considered a good competitor in the relatively substrate rich rhizosphere, the minimal number of cells was estimated to be 100. The subsequent prevalence of contaminated lettuce (internal plus external) as a result of growing in contaminated manure-amended soil was estimated using prevalence data from experiments (22). The final probability of lettuce contamination was calculated by multiplying the previous probabilities (Table 1).

Besides colonization via the roots, contamination of lettuce in the field might also occur by splash dispersal as a result of rain. The number of rain events during the growth period of lettuce was modelled by a binomial distribution (n, p), where n represented the number of growing days in the field (uniform distribution between 50 and 75 days) and p represented the average chance of rain during the growing season (6% in the Netherlands). Although it was realized that the occurrence and the magnitude of splash dispersal depend on many

factors (rain intensity, drop size, drop height etc.), the rate of success of a splash event (i.e. successfully depositing a manure derived drop on the leaf with attachment of cells and colonization as result) was estimated by expert opinion at 1% (43, 51). Because the lack of any empirical data on pathogen dynamics on or in the lettuce crop during growth in the field after attachment and colonization, the likely concentration in/on the crop at time of harvest could not be stochastically modelled. We assumed that a contaminated lettuce seedling stays contaminated up to the point of harvest.

6.2.3 Importance analysis

The Spearman correlation coefficient was used to identify critical points in the primary production chain that most significantly influence the probability of contamination and the expected level of contamination, i.e. those factors which are highly correlated with an increased probability and/or level of exposure. The model inputs were ranked based on their correlation coefficients with the output variables, obtaining an objective measure to identify possible critical control points.

6.2.4 Sensitivity analysis and risk mitigation strategies.

In order to compare the efficiency of risk mitigation options, the model input values were changed and the newly predicted contamination was calculated and compared to the baseline output. By changing one input variable at a time the relative effect on the model output was evaluated. The distributions were shifted with 50% of the mean in order to perform a sensitivity analysis. To assess the impact of several realistic risk mitigation strategies, distributions were adjusted based on available data.

1. Reducing the initial density of E. coli O157 in manure.

Some studies found diet effects which indicate there is potential for diet modifications in order to reduce *E. coli* O157 concentrations in manure. Cattle fed hay showed 75% lower *E. coli* counts in the colon compared to cattle fed grain (15). Gilbert *et al.* (26) showed a decrease of approximately 29% in *E. coli* counts in cattle faeces when fed roughage compared to grain. With respect to *E. coli* O157, sheep fed a diet characterized by a 10 to 35% fiber content showed 27% lower concentrations in their faeces compared to sheep fed a 5% fiber diet (40). The effect of a reduced density of *E. coli O157* in manure by feeding a diet higher in fiber content to cattle was evaluated by shifting the distribution describing the initial density in manure downwards by 25% of the mean.

The hypothetical reduction of 50%, as conducted for the sensitivity analysis, could in practice be reached by the use of probiotics. Addition of *Lactobacillus* strains has been shown to reduce the density of *E. coli* O157 in manure up to 63% (62).

2. Increased storage time of manure.

Longer manure storage times intuitively lead to lower pathogen load at the moment of applying manure to the field. Preventing the use of manure that is too fresh is a potential risk mitigation strategy, which is already recommended in some countries (47, 66). The effect of this strategy was evaluated by shifting the distribution for manure storage time upwards with 50%, which implicates a minimum storage time of 30 days.

***3. Reducing the prevalence of E. coli* O157:H7 in *contaminated manure*.**

Reducing farm and animal prevalence of *E. coli* O157:H7 has been subject of many studies. Although under debate, adjusting cattle diet can be a strategy to reduce the prevalence (12). Forage feeding has been found to reduce animal prevalence with 35% compared to a concentrate diet (12). To evaluate this risk mitigation strategy, the animal prevalence was reduced with 35%. Other options to reduce the prevalence of *E. coli* O157:H7 are vaccinations, probiotics, antibiotica or phage-therapy (63). These strategies could in potential reach a reduction of 50%, as used for the sensitivity analysis.

4. Increasing pathogen decline in manure.

The survival of *E. coli* O157:H7 in manure can be influenced by the physical, chemical and microbiological characteristics of the manure, which in turn is a function of cattle diet and manure type (manure versus slurry). Next to a hypothetical 50% increase in the decimal reduction time, a 42% increase was evaluated based on measured survival times in manure derived from cattle fed different diets (grass-maize silage versus straw) (21).

5. Evaluation of the 120-day interval between applying manure and harvest.

In the United States, the USDA organic certification program requires the composting of raw manure, the application to land used for a crop not intended for human consumption, or the incorporation of manure into the soil at least 90 days before harvesting an edible product that does not come into contact with the soil and at least 120 days before harvesting an edible product that does come into contact with the soil (3). With an average growing time for lettuce of 60 days, this means a time interval between applying manure and planting the lettuce seedlings of 60 days. Increasing this interval to an average of 60 days is equivalent to increasing the length of the time interval with 50%.

6. Increasing pathogen decline in manure-amended soil.

Considerable variability in survival times have been found among 36 different Dutch soils, which could be explained by the variation in levels of dissolved organic carbon per

Table 3. Reported prevalence data of *E. coli* O157:H7 on Dutch dairy farms.

No. of positive farms	No. of farms	Year	Season	Source
7	10	1996	Sept-Nov	(31)
2	34	1997	n.r.[1]	(46)
6	136	1997-1998	Year round	(30)
13	267	1998	n.r.	(46)
14	267	1998	Year round	(11)
14	163	1999	Year round	(11)
14	158	2000	Year round	(11)
13	161	1999	n.r.	(46)
1	55	2000	n.r.	(46)
11	103	2001	Year round	(11)
21	148	2002	Year round	(11)
49	678	1997-2000	Year round	(55)
13	25	2004	Aug - Sept	(19)
164	1938			

[1] Not reported

unit biomass (*DOC/biomC*) in manure-amended soils (20). This could be a potential risk mitigation strategy since *DOC/biomC* can be controlled by management choices. Next to a hypothetical 50% increase in the decimal reduction time, a 37% increase was evaluated based on the variation among the 36 soils of the earlier mentioned study. A decrease in *DOC/biomC*, resulting in an increased survival, could be achieved by amending fields with solid farm-yard manure instead of slurry (20).

7. Decreasing the aggregation parameter.

The probability of a root colliding with a manure particle depends on density of manure particles in the soil and the aggregation parameter of the negative binomial distribution which describes the spatial distribution of manure particles. A lower aggregation parameter indicates a higher level of aggregation of manure particles and subsequently a lower level of contact with roots. A higher level of aggregation could be accomplished by using solid farm-yard manure instead of slurry. The effect of a hypothetical 50% decrease in the aggregation parameter (50% increase in spatial aggregation) on the contamination of lettuce was evaluated.

6.2.5 Comparison of organic and conventional primary production.

In addition to evaluating the effects of single intervention strategies, differences in the probability of exposure were evaluated for a traditional organic and an intensive conventional scenario. Organic dairy farmers tend to feed cows a diet that is higher in fiber content. Although is has been demonstrated that this could reduce animal

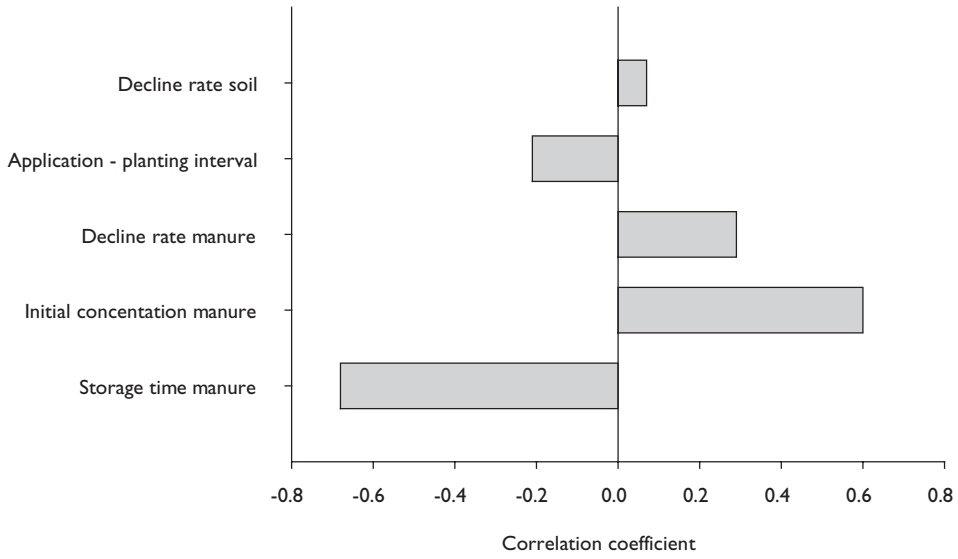

Fig. 3. Spearman rank correlation between the estimated densities of *E. coli* O157:H7 in manure-amended soil (CFU /g manure particle) and predictive factors of the exposure model.

prevalence, there is no evidence that the animal prevalence of *E. coli* O157:H7 is lower among organic dairy farms compared to conventional dairy farms (14, 19, 39). Therefore, the prevalence of contaminated manure was assumed to be identical for organic and conventional production. There is also no indication that the production system affects the concentration of *E. coli* O157:H7 in manure (17, 18). However, it has been demonstrated that the survival of *E. coli* O157:H7 in manure is a function of cattle diet (21). Refitting the survival data of Franz *et al.* 2005 (21) to the Weibull decline model revealed that the decimal reduction time was decreased with 42% when cattle were fed a diet of pure straw compared to grass-maize silage. Because the base-line model is thought to reflect the average between organic and conventional systems, the decimal reduction time was decreased with 21% for the organic system and increased with 21% for the conventional system. It was realized that feeding a diet of pure straw is an extreme organic diet and that in practice cattle are fed other fibrous bulk feed and additional concentrates. However, it has been shown that supplementation of high and low nitrogen concentrates to this diet did not influence the decline rate of *E. coli* O157:H7 significantly (21).

In addition, organic farmers tend to make more use of farm-yard manure rather than slurry to fertilize their fields in order to increase the soil organic matter content. Although it was assumed that decline rates of *E. coli* O157:H7 are higher in slurry compared to manure (47), one of the few studies which compared *E. coli* O157:H7

survival in manure and in slurry within the same experimental setting on fescue plots was unable to find a significant difference (33). The survival of *E. coli* O157:H7 in manure and slurry greatly depends on the availability of oxygen, with no difference in survival between aerobically stored slurry and manure but with a four times longer survival in manure compared to slurry when stored anearobically (57). Traditional organic dairy farms store solid farm-yard manure in heaps which are turned occasionally, creating alternating anaerobic and aerobic conditions. Conventional dairy farms produce more slurry which is stored in pits where temperatures are not exceeding 25°C and where conditions are far more anaerobic. The difference in decimal reduction time between aerobically stored manure and anearobically stored slurry in decline rate was found to be 70% (57). In practice, the manure heap will not be so aerobic as within the experimental setting of Semenov *et al.* (57) but other factors like increased temperatures and drying will additionally increase pathogen decline. The decline characteristics in the baseline model were obtained from experiments with semi-anearobically stored manure. Therefore, a traditional organic situation with respect to the decline in manure was modeled by decreasing the baseline decimal reduction time with 56% (21% for the feed and 35% for the aerobic manure storage) and a typical intensive conventional situation was modeled by increasing the decimal reduction time with 56%.

The decline of *E. coli* O157:H7 in manure-amended soil has been shown to be highly correlated with the level of dissolved organic carbon per unit biomass *DOC/biomC* (20). With the application of slurry, more *DOC/biomC* is added to soil compared with the application of solid farm-yard manure (A.V. Semenov, unpublished). The distribution for the decimal reduction time in the baseline model is based on the total set of 36 soils and is reflecting the average situation and the associated variability. The lower 50% of the distribution for the decimal reduction time in manure particles in soil was used to model the conventional situation, and the upper 50% of the distribution was used to model the organic situation. The time interval between the application of manure and planting lettuce is generally longer with organic production (field survey conducted in 2004 by the Louis Bolk Institute, Driebergen, the Netherlands). For the organic situation a Pert(14; 48; 150) was used and for the conventional situation a Pert(15; 24; 45) distribution was used. The aggregation parameter for the spatial distribution of manure particles in the soil was fixed on 0.01 for manure and 0.1 for slurry (reflecting a higher level of aggregation for manure). The amount of manure (in tons per hectare) used with the organic scenario was modeled by Pert(10; 23; 35) and for the conventional scenario by Pert(25; 28; 30), based on a field survey (field survey conducted in 2004 by the Louis Bolk Institute, Driebergen, the Netherlands). A summary of differences in variable values between the traditional organic versus intensive conventional scenarios is given in Table 4.

Table 4. Summary of changed input variables for the traditional organic and intensive conventional lettuce production scenario.

	Scenario	
Variable	Traditional organic	Intensive conventional
d_{man} Decimal reduction time of Weibull model for decline in manure (days)	Shift baseline distribution -56%	Shift baseline distribution +56%
d_{soil} Decimal reduction time of Weibull model for decline in manure in soil (days)	Lower 50% of distribution	Upper 50% of distribution
T_{soil} Time interval manure application – lettuce planting (days)	Pert(14; 48; 150)	Pert(15; 24; 45)
k Aggregation parameter of negative binomial distribution	0.01	0.1
M_{man} Quantity of manure applied to field (kg manure / ha)	Pert(10; 23; 35)	Pert(25; 28; 30)

Table 5. Results of the sensitivity analysis and the effects of intervention strategies on the density of *E. coli* O157:H7 in manure-amended soil at time of lettuce planting and the probability of exposure at time of harvesting lettuce.

Output	D_{soil}		P_{exp}	
	Mean (5^{th} and 95^{th} percentiles)	Reduction efficiency	Mean (5^{th} and 95^{th} percentiles)	Reduction efficiency
Intervention strategy	Log CFU/g	%	Log CFU/g	%
Baseline	-3.73 (-7.48 / 0.30)	-	0.34 (0.03 / 0.92)	-
Animal prevalence -35%	-	-	0.15 (0.01 / 0.51)	56
Animal prevalence -50%	-	-	0.10 (0.03 / 0.32)	65
Initial density in manure -25%	-4.35 (-8.09 / -0.31)	16	0.14 (0.01 / 0.36	59
Initial density in manure -50%	-5.01 (-8.71 / -1.15)	34	0.05 (0.01 / 0.14)	85
Storage manure +50% (min. of 30 days)	-5.11 (-8.72 / -1.11)	37	0.05 (0.01 / 0.13)	85
Decline in manure +50%	-4.59 (-8.96 / -0.25)	23	0.17 (0.01 / 0.44)	50
Decline in manure +42%	-4.53 (-8.78 / -0.30)	21	0.24 (0.02 / 0.64)	29
Manure–planting interval +50% (min. 60 days)	-4.80 (-8.48 / -0.78)	29	0.08 (0.02 / 0.18)	76
Decline in manure-amended soil +50%	-4.71 (-9.17 / -0.29)	26	0.20 (0.02 / 0.47)	41
Decline in manure-amended soil +37%	-4.52 (-8.87 / -0.19)	21	0.26 (0.02 / 0.50)	24
Spatial aggregation manure particles +50%	-	-	0.32 (0.02 / 0.86)	6
Organic production scenario	-5.36 (-10.85 / -0.36)	44	0.10 (0.01 / 0.24)	71
Conventional production scenario	- 4.04 (-6.59 / 0.73)	-8	0.89 (0.06 / 2.42)	-62

6.3 Results

6.3.1 The baseline model

Intermediate predictions of the model are the density of *E. coli* O157:H7 after storage of naturally contaminated manure and at time of planting lettuce seedlings after a time interval between manure application and planting (fig. 2). The mean density of *E. coli* O157:H7 in naturally contaminated fresh dairy manure was 2.49 log CFU/g (5^{th} percentile: 0.50, 95^{th} percentile: 5.21). At the time of applying the manure to the field (after a storage time of 61±34 days), the mean density has declined to-0.60 log CFU/g (5^{th} percentile: -4.20, 95^{th} percentile: 3.36). At the time of planting the first lettuce seedlings the mean density in the manure particles present in the soil had further declined to -3.73 (2.35, 5^{th} percentile: -7.45, 95^{th} percentile: 0.30) (fig. 2). It was deduced from the cumulative probability plot for the density in manure particles at time of planting that the chance of having more than 2 log CFU/g (which was assumed to be the critical density for root colonization) was approximately 0.6%.

Rank correlation revealed that the estimated density of *E. coli* O157:H7 in manure-amended soil at the time of planting lettuce was most highly correlated to the storage time of the manure (r = -0.68) and the initial concentration in manure (r = 0.64). (fig.3) The decline in manure (r = 0.29) and the time interval between manure application to soil and planting of lettuce seedlings (r = -0.21) showed lower correlated with the estimated density in manure-amended soil.

The modeled probability of exposure was multiplied by the number of lettuce plants on 1 hectare (111000 plants, based on 30cm distance between beds and plants) to obtain a prediction for the expected number of contaminated plants per hectare (fig. 4). The exposure model predicted 0.24 contaminated plants per hectare for root uptake (5^{th} percentile: 0.02, 95^{th} percentile 0.64) of which 0.08 (5^{th} percentile: 0.005, 95^{th} percentile 0.22) were internally contaminated. The exposure model predicted 0.10 (5^{th} percentile: 0.003, 95^{th} percentile 0.31) contaminated plants per hectare as a result of splash dispersal. In total, 0.34 (5^{th} percentile: 0.03, 95^{th} percentile 0.92) contaminated plants per hectare were predicted by the exposure model. The maximum value was 2.08 contaminated plants per hectare. The likelihood of one or more than one contaminated lettuce plants per hectare was 2.8%. For two or more contaminated plants this likelihood was 0.01%.

The probability of contamination was most highly correlated with the initial prevalence of contaminated manure (r = 0.57), the storage time of the manure (r - 0.43) and the initial concentration of *E. coli* O157:H7 in fresh manure (r + 0.38). (fig.5) The manure quantity applied (r = 0.21), the decline rate in manure (r = 0.19), the number of rain events (r = 0.17), the time interval between manure application and planting (r - 0.13), and the aggregation parameter (r = 0.11) were intermediately correlated with the probability of exposure.

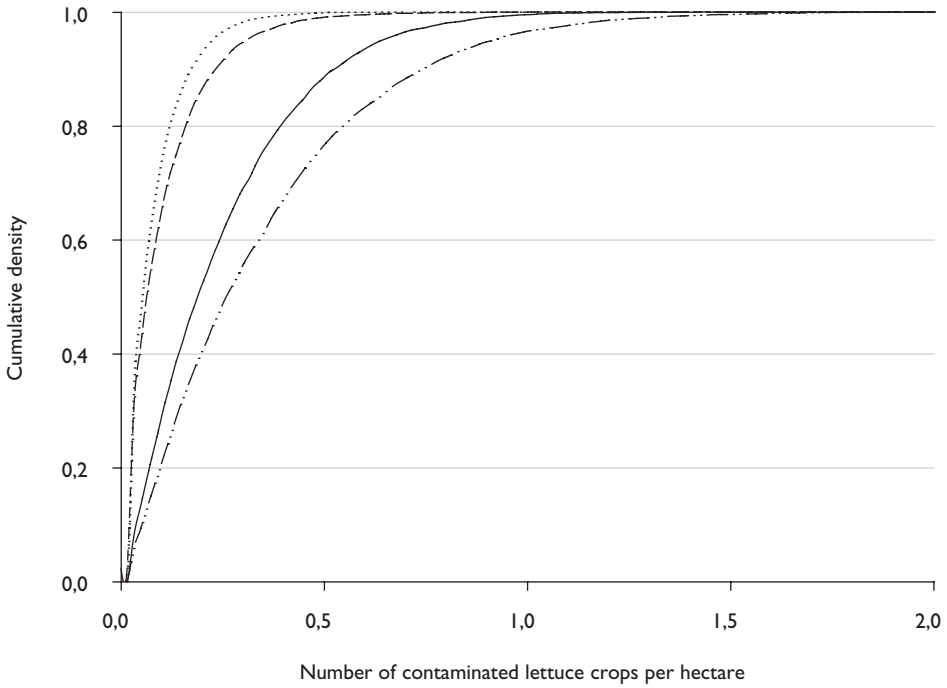

Fig. 4. Cumulative probability for the estimated number of contaminated lettuce plants per hectare by root uptake (solid line), internally contaminated plants by root uptake (dotted line), contaminated plants via splash dispersal (striped line) and contaminated crops in total (striped/dotted). The expected number of contaminated lettuce plants is deduced from multiplying the probability of exposure with the number of lettuce plants on one hectare (111000, given space between plants and between beds is 30 cm).

6.3.2 Sensitivity analysis and comparing risk mitigation strategies.

The probability of lettuce contamination was most sensitive to the storage time of manure and the initial density of *E. coli* O157:H7 in fresh manure. When the storage time was increased with 50% (resulting in a minimum of 30 days) or the initial pathogen density was decreased with 50%, the probability of contaminated lettuce was reduced with 85% to a mean probability of 0.05 contaminated lettuce plants per hectare (Table 5). Increasing the time interval between manure application and planting with 50% (to a minimum of 60 days) resulted in a reduction of 76%. Decreasing animal prevalence of *E. coli* O157:H7 with 50% lead to a reduction of 65%. Increasing the decline rate in manure or the decline rate in manure-amended soil resulted in a reduction in exposure of respectively 50% and 41%. The probability of lettuce contamination was not very sensitive to the level of spatial aggregation of manure particles (reduction of 6%).

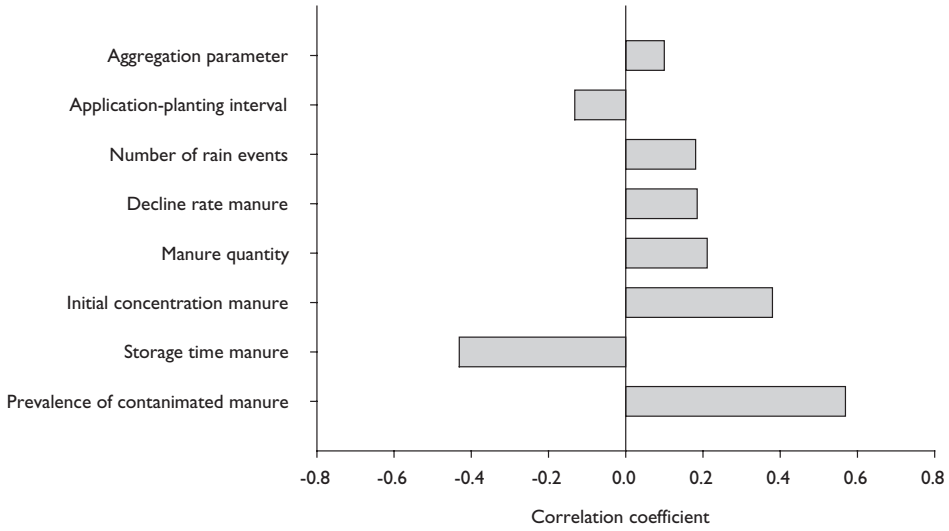

Fig. 5. Spearman rank correlation between the probability of exposure at lettuce harvest and predictive factors of the exposure model. Factors with correlation coefficient smaller than 0.1 are not shown.

In addition to the sensitivity analysis, where the distributions were shifted with 50% of their mean value, more realistic risk mitigation strategies were evaluated. Distributions were adjusted based on available data so that they reflected realistic values. The two most promising intervention strategies were increasing the manure storage time (increasing the storage time to a minimum of 30 days resulted in a decrease of 85% in exposure probability) and increasing the time interval between manure application and planting (establishing a minimum of 60 days resulted in 76% reduction in exposure probability) (Table 5). The reduction in the initial density of *E. coli* O157:H7 in fresh manure by 25% (as a result of a more fibrous cattle diet) resulted in a 59% exposure reduction. The reduction of the animal prevalence of *E. coli* O157:H7 by 35% (as a result of a more fibrous cattle diet) resulted in 56% exposure reduction. Increasing pathogen decline in manure by adjusting cattle diet to a more fibrous diet and reducing the decline rate in manure-amended soil lowering the level of dissolved organic carbon by respectively 42% and 37% resulted in 29% and 24% reduction in exposure probability. Thus, the implementation of an increased manure storage time and increased time interval between manure application and planting seem to be the most promising intervention strategies. Additional decrease in exposure can be achieved by adjusting cattle diets and soil management.

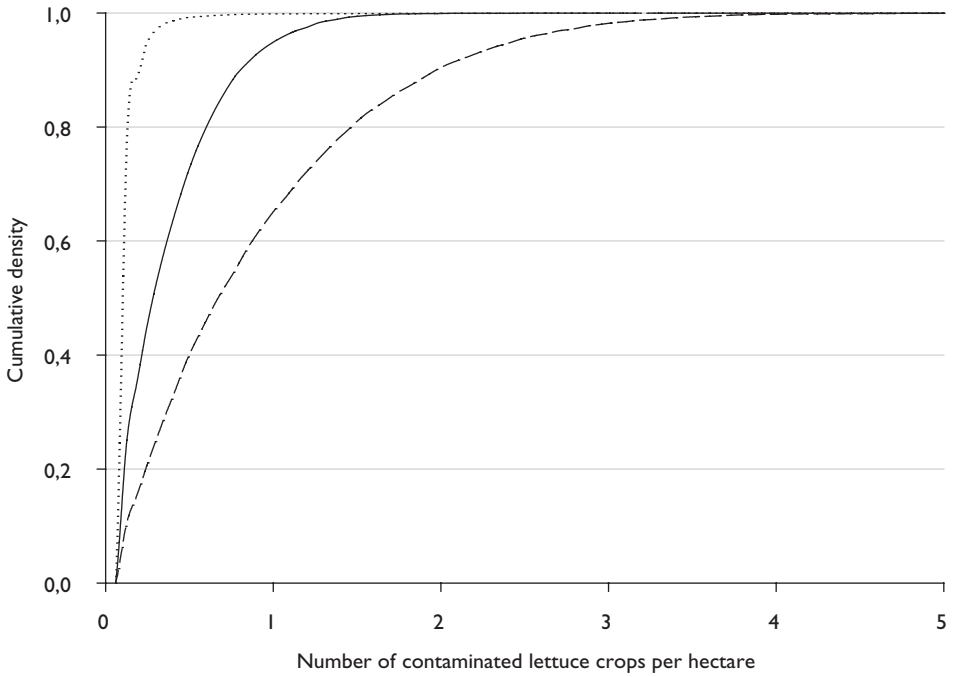

Fig. 6. Cumulative probability for the estimated number of contaminated lettuce plants per hectare for the baseline model (solid line), the organic production scenario (dotted line) and the conventional production scenario (striped line). The expected number of contaminated lettuce plants is deduced from multiplying the probability of exposure with the number of lettuce plants per hectare (111000, given space between plants and between beds is 30 cm).

6.3.2 Comparing farming scenarios: organic versus conventional.

Based on available data concerning differences in management between traditional organic and intensive conventional farming strategies (both at dairy and arable farm level) and the effects of those differences on factors influencing survival of *E. coli* O157:H7 and subsequent lettuce contamination, the exposure model was run for both farming scenario. Compared to the baseline model, the organic primary production scenario resulted in 44% reduction in *E. coli* O157:H7 density in manure-amended soil at time of planting (mean = -5.36 log CFU, 5th percentile: -10.85, 95th percentile: -0.36) (Table 5). The conventional scenario resulted in an 8% increase in density compared to the baseline model (mean = -4.04 log CFU, 5th percentile: -6.59, 95th percentile: 0.73, max = 5.72). The organic scenario resulted in a 71% reduction (mean = 0.10 contaminated plants per hectare, 5th percentile: 0.01, 95th percentile: 0.24) in the expected number of contaminated lettuce plants (fig. 7, Table 5). The conventional scenario resulted in a 62% increase in the expected number of contaminated lettuce plants (mean = 0.89 contaminated plants per hectare, 5th percentile: 0.06, 95th percentile: 2.42).

6.4. Discussion

The possible contamination of leafy greens like lettuce is of growing concern since these products are likely to be consumed raw, without any form of microbiologically lethal processing. These concerns are justified by the growing number of outbreaks and disease cases (4, 59), surveys showing the presence of human pathogens on produce (10, 29, 44) and experiments showing the persistence on plant tissue (1, 35, 45) and the possible internalization of pathogens into the edible tissue (22, 38, 61, 72).

With the present study a probabilistic exposure assessment for the contamination of lettuce by *E. coli* O157:H7 from manure-amended soil was conducted. The baseline model, which can be regarded as the average situation, estimated a presence of on average 0.34 contaminated lettuce plants per hectare. Of these, 0.24 plants were contaminated by pathogen movement in or on the plant from soil and 0.10 plants were estimated to be contaminated due to splash dispersal as a result of rain events. Approximately $65 \cdot 10^6$ lettuce heads are produced annually in the Netherlands (occupying around 600 hectares), resulting in an estimated 199 contaminated lettuce heads produced annually (1 in $3.27 \cdot 10^5$ produced heads). The upper 95th percentile would mean an estimated 538 contaminated heads annually produced in the Netherlands (1 in $1.21 \cdot 10^5$ produced heads) and the maximum of 2.08 would result in 1218 contaminated heads (1 in $5.34 \cdot 10^4$ produced heads). Conventional, intensive production of manure and lettuce was identified as a risk enhancing scenario, leading to an average 0.89 contaminated heads per hectare, which means 521 contaminated heads produced annually in the Netherlands. The upper 95th percentile indicated 1417 contaminated heads (1 in $4.59 \cdot 10^4$ produced heads) and the maximum of 5.72 would result in 3350 contaminated heads produced annually in the Netherlands (1 in $1.94 \cdot 10^4$ produced heads). Traditional organic production was found to be risk reducing, leading to only 59 contaminated heads annually produced in the Netherlands (upper 95th percentile of 141 contaminated heads). It should be stressed that the traditional organic scenario used with this exposure model can not be considered as typical mainstream organic, rather as an extreme organic management scenario. In practice, management and resulting characteristics of manure and soil are strongly overlapping for organic and conventional farms (19, 68).

Unfortunately we were not able to model the dynamics of *E. coli* O157:H7 densities in or on the lettuce plant during growth in the field to estimate the density of *E. coli* O157:H7 associated with a contaminated lettuce head at the point of harvest. Very little data is available concerning the survival, multiplication and/or death of pathogens associated with lettuce during growth in the field. It has been observed that *Salmonella* densities in surface sterilized-lettuce leaf tissue reaches levels between 3 and 4 log g^{-1} within 3 days, when grown on Hoaglands agar with a pathogen density of $1 \cdot 10^4$ to $1 \cdot 10^5$ CFU g^{-1}, where-after it stabilizes (M. M. Klerks 2007, *personal communication*).

These densities were maintained for at least 16 days, during which the shoot biomass increased 4 times. In order to maintain these densities up to the point of harvest, where the plant weight has increased approximately a thousand times (from 1 g to 1 kg), the pathogen must be able to multiply during the entire time span of plant growth (8-9 weeks), which is unlikely given the observations available. When there will be solely survival of the cells without multiplication, the pathogen density in a mature lettuce head will be not be higher than 10 CFU per g of lettuce tissue.

Although it was not the objective of this study to determine the probability of illness for a human population, we can make an estimation of the probability of disease by multiplying the probability of exposure (output of this contamination assessment) by the probability of disease from one exposure, using the dose-response envelop of Powell *et al.* (16, 52). The average probability of illness from a single lettuce consumption of 100g fresh weight was estimated at $1.29 \cdot 10^{-9}$ (5^{th} percentile: $8.95 \cdot 10^{-11}$, 95^{th} percentile: $1.41 \cdot 10^{-8}$). This is a factor 10^4 less than the estimated probability of illness from a single hamburger meal in the United States as estimated by Cassin *et al.* (13). With a constant population consumption pattern of 50 servings annually, this would result in an annual probability of illness of $6.43 \cdot 10^{-8}$ (5^{th} percentile: $2.11 \cdot 10^{-9}$, 95^{th} percentile: $1.41 \cdot 10^{-7}$), which is in the same order of magnitude as the estimated annual risk of listeriosis due to the consumption of soft cheese made from raw milk (9). However, these comparisons depend to great extent on the type of dose-response approaches used and parameter choice.

It should be stressed that in order to conduct a full risk assessment, more data are needed on pathogen dynamics in/on lettuce during growth. Pathogen densities required for internalization into lettuce and quantification of spread/survival/growth in and on the plant during growth are major knowledge gaps which need to be filled. In addition, pathogen dynamics after harvest should be incorporated. The risks could increase during post-harvest processes like slicing and the distribution of a single lettuce heads over several packages, modified atmosphere packaging and poor temperature control. Although the estimated risks are relatively low, it must be realized that *E. coli* O157:H7 is a dangerous pathogen with outbreak potential. The recent multi-state outbreak of *E. coli* O157:H7 in the USA associated with fresh packed spinach was one of the largest and deadliest outbreaks in recent times (5). It seems that the prevalence of produce-associated outbreaks is higher in the USA compared to Europe. A possible reason might be the large-scale use of irrigation water from wells in Californian lettuce/spinach production areas surrounded by hills with grazing cattle, which should be incorporated in future risk assessments.

Since post-harvest decontamination processes such as chlorination are not always effective in removing pathogens from produce, the prevention of preharvest contamination is essential for maintaining the microbiological safety of fresh produce. The present study evaluated the factors most contributing to the exposure probability

and evaluated risk mitigation strategies. Sensitivity analysis revealed that the likelihood of exposure was most sensitive to the prevalence of contaminated manure, the manure storage time and the initial density of *E. coli* O157:H7 in naturally contaminated manure. When testing intervention strategies based on realistic data, it appeared that especially increasing the manure storage time (to a minimum of 30 days) and incorporating a fertilization-to-planting interval of at least 60 days were most successful in reducing the number of contaminated lettuce heads. Although the USDA specifies a minimum fertilization-to-harvest interval of 120 days (66), Ingham *et al*. (34) concluded that the fertilization-to-planting interval was far more influencing the presence of *E. coli* on vegetables than the fertilization-to-harvest interval. Indeed, it is thought that especially lettuce seedlings are prone to contamination (36).

These time-related intervention strategies were followed by the reduction of the animal prevalence of *E. coli* O157:H7 and the initial concentration in manure. . Similarly, the risk of developing disease as a result of the consumption of *E. coli* O157:H7 contaminated beef hamburgers was also found to be very sensitive to concentration of the pathogen in manure (13). The results support the idea that efforts to implement risk mitigation strategies aimed at the delivery of safe produce to consumers should start early in the primary production chain, namely at the dairy farms.

Finally, it should be realized that *E. coli* O157:H7 is not the only STEC strain which can contaminate produce like lettuce. The overall prevalence of STEC on Dutch dairy farms is estimated to be several factors higher than the prevalence of *E. coli* O157:H7 (19). Therefore the risk of lettuce contamination by STEC in general could be several factors higher. Several strategies have been proposed to reduce both the prevalence and density of *E. coli* O157:H7 in manure, like diet modifications, vaccinations, probiotics, bacteriophage therapy and improved hygiene (7). Diet modifications have been shown to influence the survival of *E. coli* O157:H7 in manure (21), although sometimes with conflicting results (12). The use of probiotics and increased hygiene has also been proposed as potential successful strategies (62, 76, 78), but these are more difficult to implement than providing a more fibrous diet. More fibrous diets have as additional advantage that losses of nitrate into the environment are curbed (27, 53).

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Chapter 7

General discussion

7.1 Introduction

Fruits and vegetables are major components of a healthy diet, but the consumption is not per definition risk free. Fresh produce is of special concern since it is likely to be consumed raw, without any type of processing that would be lethal to harmful micro-organisms. The internationally observed increase in produce-related disease outbreaks (7, 62), surveys showing the presence of human pathogens on produce (12, 30, 49) and experiments showing the persistence on plant tissues (1, 34, 50) are suggesting that plants might be more important as a carrier for enteric pathogens than previously thought. Recent large-scale outbreaks of *E. coli* O157:H7 associated with spinach (8) and lettuce (5) in the US confirm these concerns, leading to an increased awareness of the potential microbiological risks of fresh produce.

Animal manure, that is potentially contaminated with human pathogens like *E. coli* O157:H7 and *Salmonella enterica*, is intensively used worldwide as a crop fertilizer, especially in areas where intensive livestock farming co-occur with arable farming, like in the Netherlands. Prevention of preharvest contamination is an essential part of a systems approach focused on the development of intervention strategies in order to deliver microbiologically safe vegetables to the consumer, especially since various post-harvest sanitation procedures may not be sufficient in removing pathogens (13, 22), or are not allowed. Detailed knowledge on the behaviour of pathogens in manure, manure-amended soil and edible plant tissue before harvest will contribute to the development of agricultural practices that will minimize vegetable contamination. The focus of this thesis was to identify risk factors for the survival and spread of *Salmonella enterica* and *E. coli* O157:H7 in manure and manure-amended soil, to conduct a quantitative microbial exposure assessment and to translate these results to practical suggestions for farm management.

7.2 Colonization of lettuce

7.2.1 Internalization of human pathogens

Contamination of crops in the field can occur by uptake via the root system, splash dispersal from the soil surface or directly by irrigation water. Especially the potential internal presence of pathogens is of concern since these cells will most likely not be removed by post-harvest or consumer sanitation actions, thereby posing a serious public health threat. Internalization of *Salmonella* or *E. coli* O157:H7 into the edible parts of plants has been observed for tomato (29), radish sprouts (36), bean sprouts (76), barley (43) and lettuce (65, 74).

The quantification of endophytic presence of enteric pathogens requires a thorough surface decontamination, which was applied in **Chapter 3**. *E. coli* O157:H7 and *S.* Typhimurium were found at considerable densities ($1 \cdot 10^2$ to $1 \cdot 10^4$ CFU per gram) in surface sterilized leaf tissue of lettuce seedlings grown in manure-amended soil. Interestingly, *E. coli* O157:H7 was not detected in surface sterilized leaf tissue of seedlings growing in a hydroponic system. For *Salmonella* this difference between hydroponics and soil was not observed. Probably, *E. coli* O157:H7 is more dependent on root damage and passive entrance into the lettuce plant. For *E. coli* O157:H7 the entry into the lettuce plant is most likely a passive event (64)..

Colonization of roots by rhizobacteria and foodborne human pathogens can follow similar patterns (15).The bacterial plant pathogen *Ralstonia solanacearum* has a complex and effective chemotaxis system, which is used to move to favourable conditions like those found in the rhizosphere (77). *R. solanacearum* first attaches to the root surface and forms microcolonies at locations with enhanced abundance of root exudates (57). As soon as the population density has reached a level at which plant defences can be overcome, *R. solanacearum* invades the root tissue and enters the vascular parenchyma (57) from where it can spread to the upper parts of the plant where it multiplies and clogs the xylem. It has been suggested that *Salmonella* follows the same pattern of colonization and invasion (40, 41)

7.2.2 Pre-harvest persistence of human pathogens associated with lettuce

Because of the spatial heterogeneity in physicochemical conditions, enteric pathogens may encounter microsites on plant surfaces where conditions are favourable for their growth or survival. However, *Salmonella enterica* and *E. coli* O157:H7 are likely not as well adapted as plant-colonizing bacteria to life on plant surfaces. It has been demonstrated that *S. enterica* is not able to assimilate sucrose, which is one of the

most prominent sugars present in leaf and root exudates (15). In contrast to successful colonizers of leaf surfaces, like *Pseudomonas syringae*, *S. enterica* is unable to grow on dry leaves (16). However, increased water availability and increased temperature can result in bursts of growth of *S. enterica* up to levels of $1 \bullet 10^6$ g^{-1} leaf tissue (16). The presence of epiphytic bacteria has been shown to restrict the colonization of lettuce (18). On the other hand, naturally available biofilms may afford protected colonization sites for human pathogens (23, 48).

Most studies dealing with the contamination of human pathogens into plants concerned seedlings. However, in order to become a public health threat the pathogens should be able to persist on or in the plant until harvest and up to the moment of consumption. Islam *et al.* (34, 35) demonstrated that *S.* Typhimurium and *E. coli* O157:H7 were able to persist in the lettuce phyllosphere for respectively at least three and six months, after the seedlings were planted and exposed to the pathogens via contaminated manure. *S.* Typhimurium was detected on arugula plants up to the point of harvest 17 weeks after the application of contaminated manure (50). Ingham *et al.* (32) observed mostly *E. coli*-negative lettuce samples within 100 days after manure application, but sporadic *E. coli*-positive samples for lettuce harvested 120 days after manure application. These data indicate that human pathogens can indeed persist on lettuce during the production phase up to the point of harvest.

In contrast, *E. coli* O157:H7 was not recovered from lettuce after growing in organic soil fertilized with naturally contaminated manure, neither in a field study, nor in a greenhouse experiment (37, 38). Several factors could explain the contrast with other studies. First, Cooley *et al.* (18) concluded that good agricultural practices that encourage the growth of competing bacteria may reduce the incidence of produce contamination. Indeed, organically managed soils generally show higher microbial diversity, microbial activity and microbial biomass compared to conventional soils (45, 70). These differences have been associated with an enhanced suppression of soil-borne plant pathogens (68, 69) and with lowered survival of *E. coli* O157:H7 in soil (72). Second, the active movement of pathogens towards roots based on chemotaxis to root exudates as demonstrated by Klerks *et al.* (2007, submitted) might be masked in soils high in organic matter and microbial activity (i.e. organic soils) (59). Third, relatively low pathogen densities were used (unknown natural densities in the field experiment and $1 \bullet 10^4$ CFU g^{-1} in the greenhouse experiment) (37, 38). *E. coli* O157:H7 and *S.* Typhimurium could also not be detected from lettuce grown in soils enriched with manure in which both pathogens already had declined to densities of approximately 10-100 CFU g^{-1} and 100-1000 CFU g^{-1} respectively (**Chapter 2)**. However, lettuce samples were not enriched and the pathogens could have been present in numbers below the detection limit, which might still be relevant given the very low infectious dose of *E. coli* O157:H7 (possibly 10 cells).

7.3 Exposure assessment

The exposure assessment resulted in an estimation of on average 0.10 to 0.89 contaminated lettuce crops per hectare in the Netherlands, depending on the management type **(Chapter 6)**. This estimation could be an under- or overestimation of the real exposure risks due to several reasons. The experiments described in this thesis might have underestimated the survival capabilities of *E. coli* O157:H7 (and *S.* Typhimurium) since they were conducted with green fluorescent protein-transformed (GFP) laboratory strains instead of wild-type strains. The GFP plasmid is thought to impose a metabolic burden on the cell, which might result in plasmid loss (46). The doubling time of EHEC *E. coli* was found to increase proportionally with GFP-expression, suggesting that GFP production could interfere with cell division (53). In addition, *Salmonella* growth kinetics were slightly different from those of the parent strains and were suggested to be less suitable for the development of predictive models (52). However, the survival characteristics in broth of the GFP strains used within this thesis were indistinguishable from those of the wild-type strain (13). Also the growth kinetics of three GFP-transformed *E. coli* O157:H7 strains were not different form their parent strains when tested in broth (under different pH, water activity and temperature conditions) and beef (73). The green fluorescent phenotype of *Pseudomonas fluorescence* chromosomally tagged with GFP was detectable in all growth phases, even under starvation conditions (66). The GFP-strain of *E. coli* O157:H7 used in this thesis was Shiga-toxin negative, but this was reported to make no difference in survival characteristics in manure compared to Shiga-toxin positive strains (42). The use of wild-type strains may not have given more realistic data, and survival in manure of *E. coli* O157:H7 that passed the intestinal tract of cattle was not different from the survival of the same strain directly inoculated into the manure (Scott 2006).

The possible loss of culturability of *E. coli* O157:H7 cells or weakened competitive strength for the growth on plating media might also have lead to an underestimation of survival. Direct microscopic cell counts usually were significantly higher than plate counts (A.V. Semenov, personal communication)(61). However, it is presently unknown whether these unculturable cells are weakened, have entered the so-called VBNC state and whether these cells are just as virulent as those that grow on plating media. Transfer of *E. coli* O157:H7 into the VBNC state has been shown to not only occur in response to temperature stress in aquatic environments (75), but also in environmental matrices like soil (10).

On the other hand, *E. coli* O157:H7 survival in manure and soil might have been overestimated in our exposure assessment model since the experiments used for model development were conducted at static environmental conditions of 10 or 15°C. Survival of *E. coli* O157:H7 and *Salmonella* Typhimurium significantly declined with

increasing mean temperatures and with increasing amplitude in daily temperature fluctuations (61). Thus, our model might have resulted in an overestimation of the exposure risk.

Finally, the estimated exposure risk might have been over- or underestimated because of several knowledge gaps. It was not possible to model the likelihood of the pathogen densities in or on the plant at the moment of harvest. Currently it is not known whether pathogens like *E. coli* O157:H7 or *S. enterica* can grow inside lettuce tissue up to the moment of harvest or whether they only survive or even die.

Despite these reservations about the exposure assessment in this thesis, the ultimate outcome (a probability of 10^{-4} of having a confamirated lettuce head produced in the Netherlands for conventional produced lettuce and 10^{-5} for organic produced lettuce) seems realistic in view of recent surveys carried out in the Netherlands, where 1 in 1000 lettuce heads was contaminated with *Salmonella* whereas no *E. coli* O157:H7 was found (VWA, personal communication). *Salmonella* Typhimurium does indeed survive around 10 times better than *E. coli* O157:H7 in manure **(Chapter 2)**.

Further research on pathogen dynamics in association with the edible parts of lettuce should give useful data for further modelling. There might be considerable variation in survival and potential plant contamination between *E. coli* O157:H7 isolates or between various STEC strains. Efforts should be made to study this variation so that the exposure model can be extended to STEC instead of *E. coli* O157:H7 in specific. Finally, nothing is known about how different production scenarios may impose different selective pressures which may lead to the selection of more or less virulent strains.

7.4 The need for pre-harvest risk mitigation strategies

Although the estimated risk of lettuce contamination with *E. coli* O157:H7 is not high **(Chapter 6)**, there are several reasons why the outbreak potential should not be underestimated. First, *E. coli* O157:H7 is a dangerous pathogen with an infectious dose of 50-100 cells or even less (9) and causes severe clinical symptoms like Haemolytic Uremic Syndrome (HUS). Second, the high disease burden of *E. coli* O157:H7 (31) will have significant effects on public health. Third, the prevalence and significance of non-O157 STEC strains as human pathogens is underestimated (11, 71). The Shiga toxin genes, which are a common characteristics of STEC, were detected in manure from more than 80% of the dairy farms investigated **(Chapter 4).** In Europe approximately 38% of the STEC disease cases were caused by non-O157 STEC strains (24). The number of non-O157 STEC disease cases increased with 63% between 2000 and 2005, compared to a STEC O157 disease case increase of 13%. Fourth, there are concerns regarding the efficiency and safety of post-harvest disinfection and packaging

treatments. The various sanitation procedures may not be sufficient in removing strongly attached bacteria, pathogens may be protected against removal in biofilms and within tissue structures, while internalized pathogens might not be removed or killed at all. In addition, sanitizing procedures may result in an additional risk of pathogen growth and spread. When pathogens present on the produce are not fully eliminated, while at the same time the native population of micro-organisms is strongly reduced, the competition for nutrients and space is reduced and subsequently will provide growth potential for the remaining pathogens. Finally, some large-scale *E. coli* O157:H7 outbreaks associated with leafy green vegetables like lettuce have occurred recently and the frequency seems to be increasing (5, 8, 62).

These concerns stress the importance of intervention strategies focused on the prevention of pre-harvest contamination of lettuce. Since contamination of vegetables grown in soils enriched with contaminated manure will largely depend on the survival capabilities of the pathogen in manure and manure-amended soils, this thesis identified risk factors for prolonged survival. Time is one of the most important factors in reducing *E. coli* O157:H7 densities in manure and manure-amended soils (**Chapter 6)**. By preventing application of manure that is too fresh (<30 days) and taking the application-to-planting interval into consideration (min. 60 days), risks of lettuce contamination can be drastically reduced. However, leaving production fields unplanted in spring is an economic cost and planting of lettuce takes place early in the season to avoid the risk of bolting due to high temperatures. Increasing the decline rate of human pathogens, like *E. coli* O157:H7, is therefore desirable.

7.5 Management options

7.5.1 Production of high quality manure.

Improvement of manure quality receives increased attention in order to reduce ammonia emission, nitrate leaching, stimulate microbial activity in the soil and increase soil organic nitrogen content (54, 55). This can be achieved by increasing the C/N ratio of the manure, which in turn can be achieved by feeding cattle a diet with a higher fibre content or mixing manure with straw. Survival of *E. coli* O157:H7 in manure was significantly reduced when cattle were fed a low-energy high fibrous diet (straw), compared to a high energy low fibrous diet (grass-maize silage) **(Chapter 2)**. The decline was faster in manure characterized by higher pH and higher fibre content **(Chapter 2, Chapter 4)**. When cattle are fed grains, some starch in the grain is incompletely digested and reaches the colon where it ferments and produces fermentation acids, causing the pH to drop (56). *E. coli* O157:H7 and *Salmonella enterica*

are known to be quite acid resistanct (14, 25) and may therefore encounter a selective advantage with a more acidic pH. Total *E. coli* numbers and their acid resistance were significantly increased when cattle were fed grains compared to hay (20). The digestion of diets high in fibres and low in grains leave no residual starch and subsequently result in more alkaline conditions. Manure derived from high-fibre / low-grain diets is characterized by lower levels of easily available carbon which might be an additional factor in reducing pathogen survival since *E. coli* O157:H7 is in nature a copiotrophic bacterium. Indeed, the manures that supported *E. coli* O157:H7 the best also showed the highest levels of dissolved organic carbon (DOC) **(Chapter 4)**. In addition, grain diets were demonstrated to result in decreased pH, increased *E. coli* numbers and increased concentrations of STEC virulence genes, as compared to roughage diets (27). In practice, feeding starch in the form of maize or grains is a common practice in dairy farming in order to fulfil the high energy need for high milk production. Recently, in order to reduce nitrogen losses to the environment there has been a trend in organic and more sustainable dairy farming to produce manure with higher C/N ratios, which is accomplished by feeding cattle diets with lower levels of sugars and starch and higher levels of fibres (hemicellulose, cellulose and lignin) (28, 54). In order to maintain satisfying levels of milk production some nitrogen concentrates can be fed, since they are not increasing the decline rates of *E. coli* O157:H7 and *Salmonella* Typhimurium in manure **(Chapter 2).** However, excess nitrogen in concentrates may lead to excess urea in milk and nitrogen in urine and manure. Interestingly, it has been noted that high production cows were probably more often positive for *E. coli* O157:H7, because they spent so much energy on milk production that it weakens their immune system (58).

7.5.2 Oligotrophication of agricultural soil systems

The variation on the survival of *E. coli* O157:H7 in 36 Dutch organic and conventional manure-amended soils was best explained by the level of dissolved organic carbon per unit biomass (DOC/biomC) **(Chapter 5)**. Positive relations were found between the survival and DOC/biomC, CO_2-production per unit biomass and absolute levels of dissolved organic carbon and dissolved organic nitrogen. In other words, the decline rate of *E. coli* O157:H7 was increased under more oligotrophic conditions. Soils with a high content of readily available nutrients showed a positive selection for γ-proteobacteria, being indicative of r-selection, which is selection for bacteria with potentially high growth rates like *E. coli* O157:H7 (63). In low-nutrient soil or soil with a high content of recalcitrant substrates, the percentage of *Acidobacterium* increased, being indicative of k-selection, which is selection for bacteria with lower growth potential but higher capability to compete for substrates.

The survival *E. coli* O157:H7 in soils increased in fields with a history of low-quality fertilization (artificial fertilizer and slurry) compared to high-quality fertilization (farmyard manure and compost) **(Chapter 5)**. Oligotrophication of agricultural ecosystems, which means the reduction of mineral nitrogen, soluble carbon compounds and available phosphorus, is thought to increase the natural suppression of plant diseases (69). Such a soil system can be achieved by the regular addition of organic fertilizers characterized by a relatively high C/N ratio, like solid animal manure and compost of plant or animal origin to the soil (68). *E. coli* decreased more rapidly in soils treated with solid beef cattle manure compared to soils treated with liquid swine manure (67). Slurries generally contain higher levels of DOC and DON compared to solid farmyard manure (Franz *unpublished*, (60). The application of farmyard manure to soil results in a relatively slow release of easily available nitrogen and carbon sources, and increased net nitrogen immobilization in the soil. This creates a more oligotrophic system which is a disadvantage for the copiotrophic enteric pathogens. Fertilizer of low quality (like slurry or synthetic fertilizer) would have the opposite effect since this is relatively rich in readily available carbon and nitrogen sources, which in turn can lead to eutrophication of the soil. The application of liquid manure to soil was found to bring triple the amount of water-extractable organic carbon compared to the application of solid manure (2). Moreover, urea-based and ammonium-based fertilizers temporarily solubilise soil organic matter and can induce a marked increase in dissolved organic carbon content (17). A eutrophicated soil system will not only be subjected to higher nutrient losses (54) and lower natural suppression of plant pathogens (68) but also to an increased survival of human pathogens like *E. coli* O157:H7, which implies an increased risk of spread and transfer of this pathogen into the food chain.

7.5.3 The role of biodiversity

Oligotrophication generally leads to increased biodiversity. Ecological theory argues that, at small spatial scales, diverse communities are more competitive and are more resistant to invasion than less diverse communities (21, 39). With respect to microbial communities, the invasibility of wheat rhizosphere communities by *Pseudomonas aeruginosa* was inversely related to the level of microbial diversity (47). Likewise, disease in tomato caused by *Ralstonia solanacearum* was inversely related to the complexity of the microbial community present in the rhizosphere (33). The species diversity of *Enterobactericeae* (determined with DGGE on freshly collected manure) was found to be significantly lower in those manures which were tested positive for the natural presence of *E. coli* O157:H7 (**Chapter 4).** When we assume that the microbial community in fresh manure more or less reflects the microbial community of the lower end of the gut (where *E. coli* O157:H7 is primarily located), it might indeed be that gut ecosystems

characterized by lower microbial diversity are prone to invasion by pathogens like *E. coli* O157:H7. Survival of *E. coli* O157:H7 in manure was inversely related to the absolute number of coliforms (**Chapter 4)**. These native coliforms might occupy the same ecological niche as *E. coli* O157:H7 (niche exclusion) and/or increase the level of competition for nutrients. With respect to manure-amended soil, the survival time of *E. coli* O157:H7 in organic soils was best explained by the level of dissolved organic nitrogen (positive relation) and the Eubacterial species richness (negative relation). Recently, the survival of *E. coli* O157:H7 was studied in soil microcosms in which microbial community structures were modified by different fumigation intensities (72). Survival was found to be prolonged with increased fumigation intensities. The authors argue that since the fumigation intensity was presumably inversely related to community complexity, soil systems with lowered microbial community complexity offer enhanced opportunities for *E. coli* O157:H7 survival.

Low levels of easily available nutrients and increased levels of microbial diversity have been shown to reduce the presence of *E. coli* O157:H7 and its survival in manure and soil (**Chapter 2, 4, 5**)(72). Within macro-ecology, it has been hypothesized that species richness and/or diversity will increase with increased resource heterogeneity (44) and that a community becomes more susceptible to invasion whenever there is an increase in the amount of unused (available) resources because the invader will encounter less intense competition from resident species (19). Van Elsas *et al.* (72) concluded that the most likely factor involved in the enhanced survival of *E. coli* O157:H7 was the lowering of soil microbiota complexity, which results in a lower degree of overlapping functionalities. Soil biodiversity and community complexity can be increased by oligotrophication, which can be achieved by applying manure that is high in organic matter but low in easily available carbon sources, like farmyard manure or compost (69). Subsequently this may result in a higher suppression of human pathogens.

7.6 Summary of intervention strategies.

In the United States, the USDA organic certification program requires the composting of raw manure, the application to land used for a crop not intended for human consumption, or the incorporation of manure into the soil at least 90 days before harvesting an edible product that does not come into contact with the soil and at least 120 days before harvesting an edible product that does come into contact with the soil (6). Compost must have reached a temperature between 55 and 76°C for 3-15 days (depending on the composting system). In the UK a 6 months interval between manure application and harvest was recommended (51). At European level and in the Netherlands there are, except for environmental regulations with respect to

nitrogen leaching and emission, no rules for the application of animal manure to fields used for the production of vegetables in order to minimize the risk of contamination with human pathogens. Below, a summary of intervention steps is presented aimed at reducing the risk of lettuce contamination with pathogens like *E. coli* O157:H7 (based on this thesis). These management options are proposed additional to the hygiene-code of the Horticultural Board (4) and the application of good agricultural practice (3) and may possibly be included into HACCP protocols for vegetable production. We focus on implementation in vegetable production systems like those present in the Netherlands.

1. The hygiene-code of the Horticultural Board should be followed (4).
2. In areas where vegetable farming co-occurs with livestock farming, no surface or ground water should be used for irrigation and on-farm washing purposes since this water may be contaminated with pathogens due to run-off from pastures and leaching into the groundwater.
3. Unless use is made of composted manure (at least 3-15 days with temperatures between 55 and 76°C), preferably solid manure (farmyard manure) should be applied to production fields in order create relatively oligotrophic soil conditions with relatively high levels of recalcitrant organic matter and low levels of easily available nutrients. This is strongly related to reduced survival of *E. coli* O157:H7 in manure-amended soils **(Chapter 5)**.
4. Manure derived from cattle that are fed a high energy / low fibrous diet should be avoided since survival of *E. coli* O157:H7 is prolonged in the resulting manure, as compared to manure resulting from cattle fed a low energy / high fibrous diet **(Chapter 2)**.
5. Organic growers should preferably fertilize their fields with organic cattle manure and should minimize the use of conventional manure.
6. The use of anearobically stored manure and slurry should be avoided. Survival of *E. coli* O157:H7 is especially strongly prolonged in anearobically stored manure, followed by anaerobic slurry, aerobic slurry and aerobic manure. (60).
7. Manure or slurry used should be at least 30 days old in order to prevent the application of relatively high pathogen densities to production fields **(Chapter 6)**.
8. An interval of 1-2 months between manure-application and planting of lettuce seedlings should be considered **(Chapter 6).**

Since most of the successful intervention strategies identified in this thesis typically fit a traditional organic production style, we can conclude that this production style reduces the risk of vegetable contamination compared to animal fertilizer-based conventional systems **(Chapter 6).** It should be stressed that this might not be the case when

comparing organic production with conventional systems that are based on synthetic fertilizers only. Organic vegetable production systems might also be more resistant to the invasion and subsequent spread and crop contamination due to lower levels of readily available nutrients and higher levels of biodiversity and microbial community complexity (26, 72). Sporadic introduction of *E. coli* O157:H7 in to production fields by wildlife or surface water might be halted by a higher buffering capacity against pathogen invasion and subsequent vegetable contamination. It would be desirable that future risk and exposure assessment concerning fresh produce incorporate more detailed data on pathogen-plant interactions during crop growth, the use of contaminated irrigation water and spread in the post-harvest production chain and retail.

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Summary

Summary

Salmonella enterica and Shiga-toxin producing *E. coli* (STEC) are well-known bacterial pathogens, able to cause serious disease in humans. Traditionally, disease caused by these bacteria has mostly been associated with the consumption of food products from animal origin. However, during the last decades an increase in the number of vegetable-related outbreaks of foodborne disease has been observed. Lettuce has been found to be the most implicated food-item in this product group and most of the lettuce associated outbreaks were related to *E. coli* O157:H7 and *Salmonella enterica*. The use of contaminated animal manure is considered to be a major source of potential lettuce contamination. Manure is intensively used worldwide as a crop fertilizer, especially in areas where intensive livestock farming co-occur with arable farming, like in the Netherlands. Since various post-harvest sanitation procedures may not be sufficient in removing pathogens or are not allowed, the prevention of pre-harvest vegetable contamination is an essential part of a systems approach focused on the delivery of microbiologically safe products to the consumer. Detailed knowledge on the behaviour of pathogens in manure, manure-amended soil and edible plant tissue before harvest will contribute to the development of agricultural practices that will minimize vegetable contamination. The focus of this thesis was to identify risk factors for the survival and spread of *Salmonella* Typhimurium and *E. coli* O157:H7 in manure and manure-amended soil, to conduct a quantitative microbial exposure assessment for the contamination of lettuce, and to translate these results to practical suggestions for farm management. All experiments conducted in this thesis were carried out in the laboratory with green-fluorescent-protein (GFP) labelled strains.

In **Chapter 2** the fate of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in a laboratory simulated lettuce primary production chain was assessed, as function of cattle diet, soil type and soil management. Dairy cows were fed 3 different roughage types under controlled conditions: high-digestible grass/maize silage, low-digestible grass silage and straw. Each was adjusted with supplemental high and low crude protein levels. The pathogens were inoculated into the manure, which was subsequently mixed with 2 pairs of organically and conventionally managed loamy and sandy soil. Finally, iceberg-lettuce seedlings were planted and checked for pathogens after growing. Roughage type, and not crude protein level, significantly influenced the pathogen decline rate. Although *Salmonella* Typhimurium survived better than *E. coli* O157:H7, both pathogens declined significantly faster in manure derived from a pure straw diet (higher pH, higher fibre content, lower energy content) compared to manure derived from to a high digestible grass/maize silage (lower pH, lower fibre content, higher energy content). Decline in manure derived from a pure low-digestible grass silage was intermediate. The decline rates of both pathogens were mainly determined by the

pH and fibre content of the manure (positive relations). After the first niche transition from manure to manure-amended soil both pathogens declined further and again *E. coli* O157:H7 declined faster compared to *Salmonella* Typhimurium. *E. coli* O157:H7 declined exceptionally fast in the organically managed sandy soil. After survival in manure and manure-amended soil the final, and most likely realistic, bacterial loads in the soils did not result in the presence of *E. coli* O157:H7 or *Salmonella* serovar Typhimurium in or on the edible parts of lettuce. The results of this study indicated that cattle diet and soil management are important factors with respect to the survival of human pathogens in the environment.

In order to assess whether *gfp*-tagged *E. coli* O157:H7 and *Salmonella* Typhimurium can colonize the exterior and interior of lettuce plants and up to which densities, seedlings were grown in an inoculated (10^7 cfu/g) hydroponic and soil system (**Chapter 3).** Two different morphotypes of *Salmonella* Typhimurium were used: MAE 110 (multicellular morphology) and MAE 119 (wild-type morphology). Pathogen presence was determined in surface decontaminated and untreated leaf samples. Surface decontamination with silver-nitrate was found to be superior compared to the use of sodium hypochlorite plus ethanol. With the hydroponic system internal colonization of lettuce only occurred at high densities with *S.* Typhimurium MAE 119 only. With the soil system *E. coli* O157:H7, *S.* Typhimurium 110 and *S.* Typhimurium 119 were found at considerable densities in sterilized leaf samples (respectively 3.95, 2.57 and 2.37 log CFU/g on average) with a prevalence of respectively 0.29, 0.23 and 0.15. No statistical differences were observed between the *Salmonella* morphotypes. Interestingly, a negative correlation was observed between shoot weight and leaf contamination. Although the seedlings were exposed to relatively high pathogen densities, the observed pathogen presence in lettuce seedlings after thorough surface sterilization demonstrated the possibility of their presence in locations where they are unlikely to be removed by consumer washing actions.

In order to identify risk factors for the natural presence of STEC virulence genes and prolonged pathogen survival in dairy manure, manure samples were taken from 16 organic and 9 low-input conventional Dutch dairy farms during August and September 2004 **(Chapter 4)**. The samples were screened for the presence of genes coding for Shiga toxin 1 and 2 (*stx1* and *stx2*), intimin (*eae*) and the *E. coli* O157 specific *rfbE* gene with Taqman real-time PCR. The *E. coli* O157 specific *rfbE* gene was found at 52% of the farms, while *stx1* or *stx2* was detected in 80% of the farms. Since the Shiga-toxin genes are a common characteristic of the STEC group this suggests a high prevalence of STEC strains, which should be included in future surveys. No significant differences were observed in the presence of these genes between organic and low-input conventional manure. However, relatively more low-input conventional farms tested positive simultaneously for STEC virulence genes *eaeA*, *stx1* and *stx2*. Interestingly,

the species diversity of *Enterobacteriaceae* was significantly or lower in manure positive for *rfbE*, indicating the possible important role of the microbial diversity in STEC epidemiology. This is in line with results from macroecology where ecosystems that contain a higher level of biodiversity are les susceptible to biological invasion. The survival of a GFP-expressing *E. coli* O157:H7 was determined in all 25 manures and described by a biphasic decline model. The overall survival time was negatively related to the numbers of generic coliforms present in the manure (indicating the potential role of competition with the native microflora) and the pH (which is identical to the results obtained in Chapter 1).

Manure-amended agricultural soil is an important part in the primary production chain of lettuce with respect to the potential pathogen transfer from manure to crops. The aim of the study presented in **Chapter 5** was to identify risk factors for *E. coli* O157:H7 persistence in manure-amended soil. The survival of a GFP-expressing *E. coli* O157:H7was determined in a set of 36 Dutch arable soils, which constituted 18 pairs of neighbouring organic and conventional soils with identical soil type (sand or loam). Inoculated manure was added to the soils and pathogen survival was determined by dilution plating on selective medium. Additionally, several biotic and abiotic manure-amended soil characteristics were determined. The Weibull model, which is the cumulative form of the underlying distribution of individual inactivation kinetics, proved to be a suitable model for describing the decline of *E. coli* O157:H7. Pathogen survival ranged from 54 to 105 days and the variability in survival times followed a logistic distribution. Due to large variation among soils of each management type, no differences were observed between organic and conventional soils. Although the initial decline was faster in sandy soils, no significant differences were observed in survival time between sandy and loamy soils. Multiple regression analysis was used to identify those factors which explain most of the variation in the survival time. For sandy, loamy and conventional soils the variation in survival time was best explained by the level of dissolved organic carbon per unit biomass, with prolonged survival at increasing levels of dissolved organic carbon per unit biomass. For organic soils the variation in survival time was best explained by the level of dissolved organic nitrogen (positive relation) and the microbial species diversity as determined by DGGE (negative relation). Survival increased with a field history of low-quality manure (artificial fertilizer and slurry) compared to high-quality manure application (farmyard manure and compost). It was concluded that *E. coli* O157:H7 populations decline faster under more oligotrophic soil conditions, which can be achieved by the use of organic fertilizer with a relatively high C/N ratio and consequently a relatively low rate of nutrient release.

In **Chapter 6** a quantitative microbial exposure assessment is presented to estimate the number of *E. coli* O157:H7 contaminated lettuce crops per hectare at the moment of harvest. As much as possible Dutch data was used to reflect the

Dutch primary production chain of lettuce. The transmission of *E. coli* O157:H7 was modeled probabilistically through the primary production chain of lettuce, taking the pathogen prevalence and densities in different ecological habitats into account (cattle, manure, manure-amended soil and lettuce). The baseline model estimated an average presence of 0.34 (5^{th} percentile: 0.03, 95^{th} percentile 0.92) contaminated lettuce plants per hectare (on average 1 in $3.27 \cdot 10^5$ produced heads in the Netherlands). Sensitivity analysis revealed that the likelihood of exposure was most sensitive to the prevalence of contaminated manure, the manure storage time and the initial density of *E. coli* O157:H7 in naturally contaminated manure. Testing these hypothetical intervention strategies with realistic data revealed that the incorporation of a minimum manure storage time of 30 days and a minimum fertilization-to-planting interval of 60 days, were most successful in reducing the number of contaminated lettuce heads. A traditional organic production scenario resulted in a 71% reduction in the expected number of contaminated lettuce plants (0.10 contaminated plants per hectare, 5^{th} percentile: 0.01, 95^{th} percentile: 0.24), while an intensive conventional scenario resulted in an increase of 62% (0.89 contaminated plants per hectare, 5^{th} percentile: 0.06, 95^{th} percentile: 2.42).

Finally, an extensive discussion concerning chapters 2 to 6 is described in **Chapter 7**. This includes suggestions for future research, the reliability of the exposure model, the role oligotrophication and biodiversity in pathogen suppression, and management options to minimize the risk of lettuce contamination by human pathogenic bacteria. It was concluded that management strategies that lead to lower levels of easily available carbon sources and/or increased microbial diversity in manure and manure-amended soil (oligotrophication) are reducing the risks of pathogen survival and subsequent lettuce contamination. This can be achieved by producing and using manure with a relatively high C/N ratio, like solid farmyard manure or compost.

Samenvatting

Samenvatting

Salmonella enterica en Shiga toxine-producerende *E. coli* (STEC) staan bekend als pathogenen die in staat zijn om ernstige ziekte te veroorzaken bij mensen. Ziekte veroorzaakt door deze bacteriën werd voorheen voornamelijk toegeschreven aan de consumptie van voedsel van dierlijke oorsprong. Echter, gedurende de laatste decennia is er een toename waargenomen in het aantal uitbraken van voedselvergiftiging gerelateerd aan de consumptie van verse groenten. Het is gebleken dat sla het meest frequent betrokken groenteproduct is en dat de meeste uitbraken gerelateerd aan sla werden veroorzaakt door *E. coli* O157:H7 en *Salmonella enterica*. Het gebruik van besmette dierlijke mest wordt beschouwd als een van de belangrijkste oorzaken van de besmetting van groenten zoals sla. Dierlijke mest wordt wereldwijd intensief gebruikt als meststof voor de productie van voedselgewassen, met name in gebieden waar ook intensieve veehouderij voorkomt. Omdat verschillende ontsmettingsprocedures na de oogst niet altijd voldoende efficiënt zijn in het verwijderen van pathogenen of niet zijn toegestaan, is de preventie van besmetting gedurende de primaire productie fase een essentieel onderdeel van een systeembenadering gericht op het leveren van microbiologisch veilige producten aan de consument. Gedetailleerde kennis omtrent het gedrag van pathogenen in mest, met mest verrijkte bodem en gewas zal bijdragen aan de ontwikkeling van landbouwpraktijken die het risico op de besmetting van groenten minimaliseren. De nadruk van dit proefschrift lag op het identificeren van risicofactoren voor de overleving en verspreiding van *Salmonella* Typhimurium en *E. coli* O157:H7 in mest en met mest verrijkte bodem, het ontwikkelen van een kwantitatief microbieel risicomodel voor de besmetting van sla met deze pathogenen, en het vertalen van de resultaten naar praktische aanbevelingen voor de praktijk. Alle experimenten binnen dit proefschrift zijn uitgevoerd in het laboratorium met stammen die het gen bezitten om een zogenoemd groen-fluorescerend eiwit (GFP) te produceren.

In **Hoofdstuk 2** is de overleving van *Salmonella* Typhimurium en *E. coli* O157:H7 bestudeerd gedurende een in het laboratorium nagebootste primaire productieketen van sla, als functie van melkveevoederregime, bodemtype, and bodemmanagement. Melkveekoeien kregen, onder gecontroleerde condities, drie verschillende soorten ruwvoer gevoerd: goed verteerbaar maïs- en graskuil, slecht verteerbare graskuil en stro. Elk van deze diëten werd aangevuld met supplementen met hoge en lage eiwitgehalte. De pathogenen werden geïnoculeerd in de mest, welke vervolgens werd gemixt met twee paren biologische en gangbare leem- en zandgronden. Ten slotte werden sla-zaailingen geplant en gescreend voor de aanwezigheid van *Salmonella* Typhimurium en *E. coli* O157:H7. De overleving van deze bacteriën in mest werd significant beïnvloed door het ruwvoer type en niet door het eiwitgehalte. Ondanks dat *Salmonella* Typhimurium beter overleefde dan *E. coli* O157:H7, verdwenen beide pathogenen

significant sneller in mest afkomstig van het strodieet (hogere pH, hoger vezelgehalte, laag energiegehalte) vergeleken met mest afkomstig van het goed verteerbaar maïs- en graskuildieet. Afdoding in mest afkomstig van het slecht verteerbare graskuil dieet was intermediair. De afdodingssnelheid van beide pathogenen werd voornamelijk bepaald door de pH en het vezelgehalte van de mest (positieve relaties). Na de eerste niche transitie van mest naar met mest verrijkte grond zette de afdoding van beide pathogenen zich voort en wederom verdween *E. coli* O157:H7 sneller dan *Salmonella* Typhimurium. De *E. coli* O157:H7 populatie nam exceptioneel snel af in de biologische zandgrond. Na overleving in mest en met mest verrijkte grond leidde de uiteindelijke, en waarschijnlijk realistische, aantallen pathogenen aanwezig in de met mest verrijkte grond niet tot de aanwezigheid van *Salmonella* Typhimurium en *E. coli* O157:H7 in de eetbare delen van de slaplanten. De resultaten van deze studie tonen aan dat het dieet van melkvee en bodemmanagement belangrijke factoren zijn met betrekking tot de overleving van humaanpathogenen in het landbouwmilieu.

Om te bekijken of de GFP-stammen van *E. coli* O157:H7 en *Salmonella* Typhimurium in staat zijn om het uit- en inwendige van slaplanten te koloniseren en tot op welke dichtheden dit gebeurt, werden zaailingen opgegroeid in een geïnoculeerd (10^7 cfu/g) hydroponisch systeem en op geïnoculeerde grond **(Hoofdstuk 3)**. Twee verschillende morfotypen van *Salmonella* Typhimurium werden gebruikt: MAE 110 (multicellulair morfotype) en MAE 119 (wild-type morfologie). De aanwezigheid van de pathogenen werd vastgesteld in oppervlakte-gedecontamineerde en onbehandelde bladmonsters. Oppervlakte-decontaminatie met zilvernitraat was superieur vergeleken met natrium-hyochloride en ethanolbehandeling. In het hydroponische systeem vond interne kolonisatie alleen plaats in hoge dichtheden met *S.* Typhimurium MAE 119. Bij zaailingen gegroeid op besmette grond werden alle drie de pathogenen in behoorlijke dichtheden aangetoond in oppervlakte-gedecontamineerde bladmonsters (respectievelijk gemiddeld 3.95, 2.57 en 2.37 log CFU/g), met een prevalentie van respectievelijk 0.29, 0.23 en 0.15. Er waren geen statistische verschillen tussen de *Salmonella* morfotypen. Opvallend was de negatieve correlatie tussen het bovengrondse plantgewicht en de mate van bladkolonisatie. Ondanks dat de zaailingen werden blootgesteld aan relatief hoge pathogeendichtheden, tonen deze resultaten aan dat de betreffende pathogenen aanwezig kunnen zijn in locaties op of in het blad waar ze hoogstwaarschijnlijk niet kunnen worden verwijderd door het wassen van de consument.

Om risicofactoren te identificeren voor de natuurlijke aanwezigheid van STEC virulentiegenen en langdurige overleving in mest, zijn mestmonsters verzameld van 16 biologische en 9 "low-input" gangbare melkveebedrijven gedurende augustus en september 2004 **(Hoofdstuk 4)**. De samples werden getest met Taqman real-time PCR op de aanwezigheid van genen die coderen voor Shiga-toxine 1 en 2 (*stx1* en *stx2*),

intimine (*eae*) en het *E. coli* O157-specifieke *rfbE*-gen. Het *rfbE*-gen werd gevonden op 52% van de bedrijven en *stx1* of *stx2* zelfs op 80% van de bedrijven. Omdat de Shiga-toxine genen een gemeenschappelijk kenmerk zijn van de STEC groep, suggereert dit een hoge prevalentie van STEC stammen. Dit zou in ogenschouw moeten worden genomen gedurende verdere surveys. Er werd geen significant verschil aangetoond in de aanwezigheid van virulentiegenen tussen biologische en "low-input" gangbare melkveebedrijven. Echter, relatief meer "low-input" gangbare melkveebedrijven werden positief bevonden voor *stx1*, *stx2* en *eae*. Opvallend was dat de *Enterobacteriaceae*-soorten rijkdom significant lager was in mestmonsters die positief waren voor het *rfbE* gen. Dit sluit aan bij resultaten uit de macro-ecologie, waar ecosystemen met een hogere biodiversiteit minder gevoelig zijn voor biologische invasie. De overleving van (GFP) *E. coli* O157:H7 is bestudeerd in all 25 mesten en beschreven door een zogenaamde "biphasic" overlevingsmodel. De overleving bleek negatief gecorreleerd met de het aantal coliform bacteriën (duidend op de potentiële rol van competitie met de natuurlijk aanwezige microflora) en de pH (wat identiek is aan de resultaten uit Hoofdstuk 1).

Wat betreft de potentiële pathogeenoverdracht van mest naar sla, is met mest verrijkte landbouwgrond een belangrijke schakel in de primaire productieketen van sla. Het doel van de studie beschreven in **Hoofdstuk 5** was het identificeren van risicofactoren voor de overleving van *E. coli* O157:H7 in met mest verrijkte grond. De overleving werd vastgesteld in een set van 36 Nederlandse landbouwgronden, bestaande uit 18 paren biologische en gangbare gronden met identiek bodemtype (zand of leem/klei). Geïnoculeerde mest werd gemengd met de gronden en de overleving van *E. coli* O157:H7 werd vastgesteld door verdunningsreeksen en uitplaten op selectief medium. Daarnaast werden verschillende biotische en abiotische bodemkarakteristieken vastgesteld. Het Weibull-model, wat een cumulatieve vorm is van de theoretische onderliggende verdeling van individuele afdodings-karakteristieken, bewees een geschikt model te zijn om de overleving te beschrijven. Overleving liep uiteen van 54 tot 105 dagen en de variabiliteit in overleving volgde een logistische distributie. Door de grote variatie tussen de verschillende gronden werd geen verschil in de overleving gevonden tussen biologische en gangbare gronden. Alhoewel de initiële afdodingssnelheid hoger was in zandgronden, was er geen verschil in uiteindelijke overlevingstijd van *E. coli* O157:H7 tussen zand- en leem/kleigronden. Meervoudige regressie is gebruikt om de factoren te identificeren die de meeste variatie in overlevingstijd bepalen. Voor zand, leem/klei en gangbare gronden werd deze variatie het best verklaard door het gehalte aan opgeloste organische koolstofverbindingen per eenheid biomassa koolstof, waarbij de overleving toeneemt met een hoger gehalte aan deze substraten. Bij biologische gronden werd de overlevingstijd voornamelijk bepaald door het gehalte aan opgeloste organische stikstof verbindingen (positieve relatie) en de microbiele soorten rijkdom zoals bepaald met DGGE (negatieve relatie). De overleving was langer bij een veldhistorie gekenmerkt

door het gebruik van mest van lage kwaliteit (kunstmest en drijfmest), vergeleken met een veldhistorie gekenmerkt door gebruik van mest van hoge kwaliteit (vaste mest en compost). Er kon worden geconcludeerd dat *E. coli* O157:H7 populaties sneller afnemen bij meer oligotrofe bodemcondities. Dit zou kunnen worden bereikt door het gebruik van mest met een relatief hoge C/N ratio en dus een relatief langzame afgifte van nutriënten.

In **Hoofdstuk 6** is een kwantitatief microbiologische blootstellingsmodel ontwikkeld om het aantal met *E. coli* O157:H7 besmette slakroppen per hectare in te schatten. Hierbij is de Nederlandse primaire productieketen van sla beschouwd en is zoveel mogelijk Nederlandse data gebruikt om het model te parametriseren. De transmissie (prevalentie en dichtheid) van *E. coli* O157:H7 door de verschillende agro-ecologische habitats van de primaire productieketen (melkvee, mest, met mest verrijkte grond en sla) is probabilistisch gemodelleerd. Het baseline model schatte een gemiddelde prevalentie van 0.34 (5^{e} percentiel: 0.03, 95^{e} percentiel: 0.92). besmette slakroppen per hectare (gemiddeld 1 op de $3.27 \cdot 10^{5}$ in Nederland geproduceerde slakroppen). Een gevoeligheidsanalyse toonde aan dat de hoogte van de prevalentie van besmette slakroppen het meest gevoelig was voor de prevalentie van besmette mest (m.a.w. de prevalentie van besmette melkveebedrijven), de opslagtijd van mest en de initiële dichtheid van *E. coli* O157:H7 in mest. Wanneer deze hypothetische interventiestrategieën werden getest in het model door de verdelingen te re-parametriseren bleek dat het instellen van een minimum bewaartijd voor mest van 30 dagen en een minimum tijdsinterval tussen het aanbrengen van mest en het planten van slaplanten van 60 dagen de meest succesvolle interventiestrategieën waren. Een traditioneel biologische productiescenario resulteerde in een reductie van het aantal besmette slakroppen van 70% (gemiddeld 0.10 per hectare, 5^{e} percentiel: 0.01, 95^{e} percentiel: 0.24), terwijl een intensief gangbaar scenario resulteerde in een toename van 62% (gemiddeld 0.89 per hectare, 5^{e} percentiel: 0.06, 95^{e} percentiel: 2.42).

Hoofdstuk 7 presenteert een uitvoerige discussie betreffende hoofdstuk 2 tot en met 6. Deze omvat suggesties voor toekomstig onderzoek, de betrouwbaarheid van het model, de rol van oligotroficatie en biodiversiteit in het onderdrukken van humaan pathogenen en management opties om het risico op besmetting van sla met humaan pathogenen te minimaliseren. Er werd geconcludeerd dat managementstrategieën die leiden tot lagere gehalten aan makkelijk opneembare koolstofbronnen en/of verhoogde microbiële diversiteit in mest en met mest verrijkte grond (oligotroficatie) risico verlagend zijn met betrekking tot pathogeenoverleving en de daaropvolgende potentiële besmeting van sla. Dit kan worden bereikt door de productie en gebruik van mest met een relatief hoge C/N ratio, zoals vaste mest (potstalmest) en/of compost.

Nawoord

Nawoord

Januari 2003. Vriend en studiegenoot David Cohen mailt mij een advertentie door voor een promovendus bij Biologische Landbouwsystemen (BFS) die zou moeten gaan werken aan de microbiologische veiligheid van verse groenten. "Iets voor jou?". Ondanks dat landbouw en microbiologie weinig aan bod waren gekomen gedurende mijn biologie studie in Utrecht, zag ik hier de kans om praktijk gericht onderzoek te gaan doen. Bovendien sprak de combinatie van experimenteel werk en het modelleren van risico's mij erg aan. Ik kon aan de slag binnen een projectteam bestaande uit 3 promovendi, een postdoc en een technisch assistent. Vier jaar en 3 maanden later lever ik de leesversie van mijn proefschrift in; ruim 210 pagina's en 7 hoofdstukken. Dit had ik absoluut niet voor elkaar kunnen krijgen zonder de assistentie, hulp en medeleven van collega's, vrienden en familie.

Allereerst wil mijn supervisie team bedanken: promotor Ariena van Bruggen en co-promotor Aad Termorshuizen. Ariena, mede dankzij jouw intensieve begeleiding is dit proefschrift succesvol in iets meer dan vier jaar afgerond. Ik heb bewondering voor je tomeloze enthousiasme en betrokkenheid. Je hebt me veel geleerd over microbiologie, phythopathologie, statistiek, biologische landbouw en spijkers met koppen slaan. Bedankt daarvoor! Aad, bij jou kon ik altijd binnenlopen met zelfs de kleinste vragen. Alhoewel die kleine vraagjes dan soms toch veel ingewikkelder waren dan ik dacht en we er soms ook niet helemaal uitkwamen, waren deze ontspannen discussies vaak uiterst leerzaam voor mij. Misschien schrijven we nog wel eens samen een publicatie over de biologische interpretatie van log-getransformeerde data? Naast het werk was je ook een fijne gesprekspartner wat betreft reizen en buitensport, passies die we gemeen bleken te hebben. Ik hoop dat dit nog lang zo blijft!

Vanaf mijn eerste dag als promovendus ben ik onder de hoede genomen van Anne van Diepeningen, postdoc bij BFS. Anne, dankzij jou kon ik vrijwel meteen aan de slag met een groot experiment. In razend tempo heb je mij enorm veel geleerd over microbiologie, moleculaire biologie en het werken met de toch gevaarlijke bacteriën *E. coli* O157:H7 en *Salmonella* Typhimurium. Jouw hulp bij het opzetten en uitvoeren van experimenten was werkelijk onmisbaar. Daarnaast was je ook een erg gezellige kamergenoot, iets wat ik na je vertrek vaak heb gemist.

Ik heb het als een enorm voordeel ervaren om in een project te werken met twee andere promovendi. Michel, je bent iets eerder gestart aan je promotie-onderzoek binnen dit project en je bent ook iets eerder gepromoveerd. Dr. Klerks, ik begreep weinig van de moleculaire detectiemethoden die je aan het ontwikkelen was maar dat heb je me aardig bij weten te brengen. Je hebt me er zelfs mee leren werken, wat nodig was voor deel van mijn onderzoek. Ik wil je bedanken voor de hulp die we elkaar hebben gegeven tijdens ons onderzoek, wat ook geresulteerd heeft in gezamenlijke publicaties. De lange dagen

slaplantjes malen en *Salmonella* uitplaten staan nog in mijn geheugen gegrift! Naast het harde werken hebben we het ook vaak erg gezellig gehad: tijdens mestmonsters halen bij de boer, in Wenen bij het ISME symposium en zomaar op momenten tussendoor. Ik kijk uit naar toekomstige samenwerking en meer gezellige tijden! Sasha, you started one and a half year later with your research within this project. You were before at BFS as a master-student to work with Gerbert and you got the opportunity to work as a PhD student at BFS. I admire your strength to leave your home and family in Russia and move to Wageningen. Thanks for your help with experiments, discussions, table-tennis games, lunches, playing pool and drinking vodka. I also enjoyed very much our time in Melbourne, where we participated in the VTEC symposium.

Oscar en Hennie, zonder jullie waren een aantal van de belangrijkste resultaten uit dit proefschrift nooit boven water gekomen. Oscar, ik weet niet hoe je het doet maar jouw DGGE's zijn altijd super. Bedankt voor alle tijd en inzet die je daar aan hebt besteed. Hennie, we hebben veel gelachen maar je hebt ook zeer belangrijke data voor mijn proefschrift vergaard: de chemische analyses van mest- en bodemmonsters. Ik heb altijd erg genoten van het kopje koffie wat we regelmatig 's ochtends vroeg bij jou op de kamer deden samen Annie en Joke van TPK. Ook Wampie en Lien, de secretaressen van BFS, wil ik bedanken voor de gezellige tijd en hun ondersteuning in allerlei zaken. Andre, je was een zeer gezellige buurman!

Ik heb het geluk gehad dat er redelijk wat studenten geïnteresseerd waren in het onderzoek aan humaan pathogene bacteriën. Zij hebben behoorlijk wat werk verricht wat opgenomen is in verschillende delen van dit proefschrift. Wouter, de geslotenheid van de verschillende schakels in de productieketen heeft het jou erg moeilijk gemaakt om een kwantitatief overzicht te krijgen in de productieketen maar uiteindelijk heb je ons aardig op weg geholpen. Anna, je hebt uitstekend werk gedaan aan de kolonisatie van slaplanten met *E. coli* O157:H7 en *Salmonella* Typhimurium, wat direct heeft geleid tot een publicatie. Arjan, jij was zo zelfstandig dat ik je helemaal kon laten gaan. In eerste instantie heb je gewerkt aan de effecten van antibiotica, geproduceerd door *Pseudomonas fluorescence*, op *E. coli* O157:H7 en *Salmonella* Typhimurium (Jos Raaijmakers bedankt voor je hulp en de stammen). De groei van de pathogenen werd in vitro inderdaad geremd door deze antibiotica. Onze verbazing was groot toen bleek dat slaplanten vele malen meer gekoloniseerd werden door *E. coli* O157:H7 en *Salmonella* Typhimurium in de aanwezigheid van antibiotica producerende *Pseudomonas* dan wanneer deze afwezig waren. Dit experiment behoeft nog steeds een herhaling! Vervolgens heb je een uitgebreid protocol ontwikkeld om met behulp van flow-cytometry te bekijken wat er met *E. coli* en *Salmonella* gebeurt gedurende overleving in de bodem. Uiteindelijk had je geen tijd meer om hier een echt experiment mee te doen omdat je een stage-afspraak had bij de NASA. Ik hoop dat alles goed is verlopen daar. Stefan, de Westlander in hart en nieren, jij hebt een gigantische hoeveelheid praktisch werk verricht dat ik

je het bijna niet meer kon vragen om het ook nog te analyseren. Toch ben je een heel eind gekomen en heeft jouw dataset geleid tot 2 publicaties, en wellicht zelfs een derde. Uitzonderlijk! Ik zal onze gezellige dagen rondrijden door Nederland om bodemmonsters te verzamelen niet snel vergeten.

Naast alle directe hulp van collega's en studenten, heb ik indirect veel steun gehad aan familie en vrienden. Jochem, de dagelijks reis naar Wageningen (soms met trein en fiets, soms met de auto en soms helemaal op de fiets) werd een stuk dragelijker met jouw gezelschap. Met onze tijd bij het NIOO erbij gerekend lijkt het alsof het nooit anders is geweest. We moeten zeker onze doordeweekse fietstochtjes van Utrecht naar Wageningen en de MTB-tochtjes tijdens de weekenden erin houden! Ik wil ook al mijn vrienden bedanken. Ondanks dat jullie je niet direct nuttig hebben gemaakt betreffende mijn proefschrift (wat ging over poep in planten toch?), heb ik mede dankzij jullie de afgelopen jaren kunnen ontspannen, genieten en afzien. Thanks!

Hans en Lida, uiteindelijk hebben jullie het voor een groot deel mogelijk gemaakt voor mij om te gaan promoveren door de steun die ik altijd heb gekregen. Bij jullie heb ik altijd ongeremd kunnen vertellen over alles wat mij bezig hield, van bergsport en expedities tot studie en promotie. Bedankt daarvoor!

Lieve Joke, de afgelopen jaren zijn naar mijn gevoel razendsnel voorbij gegaan en er is heel veel gebeurd. Na de fantastische drie maanden reizen, gingen we eindelijk echt samenwonen in Utrecht en een paar maanden later kon ik aan de slag in Wageningen. Soms was ik weleens wat afwezig doordat ik thuis nog met mijn hoofd bij mijn werk was maar daar heb je nooit over geklaagd. Halverwege mijn promotietraject ging ik voor een maand op klimexpeditie naar Chili, wat voor jou geen gemakkelijke tijd was in de donkere december dagen. Maar gelukkig hebben we ook heel veel samen kunnen genieten, zowel thuis als op reis. Ik heb enorm genoten van de tripjes naar congressen waarbij je altijd met me mee ging zodat we aansluitend een mooie vakantie konden houden: het ISME congres in Cancun en het VTEC congres in Melbourne. Na het ISME congres in Wenen kwam je speciaal een weekend om mijn verjaardag daar te vieren. Ons leven kwam langzaam in een stroomversnelling. Gedurende de laatst genoemde trip was je ruim 4 maanden zwanger en hebben we na het congres 3 weken door Tasmanie getrokken, een memorabele vakantie! Op 16 maart 2007 werd Eline geboren en werden we vader en moeder van een prachtig meisje. Nog geen vier maanden later traden we, na bijna 9 jaar samen, in het huwelijk. Ondertussen had ik alweer een nieuwe baan gevonden bij het RIKILT in Wageningen en hebben we een huis gekocht in Wageningen. Wie weet wat ons allemaal nog te wachten staat!?

Lieve Eline, je bent geboren terwijl ik zat te zwoegen om mijn proefschrift af te krijgen. Je was echter net zo'n slaapkop als je vader en moeder en je hield ons slechts sporadisch wakker 's nachts. Jouw opgewekte humeurtje en je lach (die je vaak laat zien!) maken me intens gelukkig. Ik hoop dat dit nog lang zo mag blijven!

Curriculum vitae

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Eelco Franz werd geboren op 27 augustus 1977 te Delft. In 1995 behaalde hij zijn VWO diploma aan het Hugo Grotius college te Delft. Van 1995 tot 2002 studeerde hij Biologie aan de Universiteit Utrecht. Zijn eerste afstudeerproject betrof experimenteel evolutionair onderzoek naar de interactie tussen temperatuur en voedselkwaliteit op de ontwikkeling van de fruitvlieg *Drosophila melanogster*, uitgevoerd bij de vakgroep Evolutionaire Populatiebiologie aan de Universiteit Utrecht. Vervolgens werd een tweede afstudeerproject uitgevoerd aan het Centrum voor Terrestrische Ecologie van het Nederlands Instituut voor Ecologisch Onderzoek (NIOO) te Heeteren. Hier werd samen met een medestudent een computermodel ontwikkeld om het effect van een fluctuerend milieu op het behoud van genetische variatie te onderzoeken. Na een jaar werken en reizen startte hij in 2003 met zijn promotieonderzoek bij de leerstoelgroep Biologische Landbouwsystemen van de Wageningen Universiteit. Daar verrichtte hij onderzoek naar de microbiële ecologie van de humaan pathogene bacteriën *Escherichia coli* O157:H7 en *Salmonella enterica* serovar Typhimurium in de primaire productieketen van sla. De data werd gebruikt om een kwantitatief risicomodel te maken. De resultaten hiervan zijn terug te vinden in dit proefschrift. Tijdens zijn promotieonderzoek zijn drie grote internationale congressen bezocht waar de resultaten werden gepresenteerd. Per 1 augustus 2007 is hij werkzaam als wetenschappelijk onderzoeker bij het RIKILT – Instituut voor Voedselveiligheid, te Wageningen.

Publication list

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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 (6 credits)

- Risk analysis of human pathogen spread in the vegetable production industry: a comparison between organic and conventional production chains (2003)

Laboratory Training and Working Visits (0.3 credits)

- Modeling food safety risks with Bayesian techniques, (2006)

Post-Graduate Courses (10 credits)

- Quantitative Microbial Risk Assessment, MG3S (2003)
- Advanced Statistics, PE&RC (2004)
- Science of Organic Production (+organizing) PE&RC (2005)

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

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- Bacteriology meetings (2003-2007)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (3 credits)

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- NWO/WUR workshop Human Pathogens (+oral) (2003)
- PE&RC annual meeting "Biological disasters" (2005)
- WUR workshop manure quality (+oral) (2005)
- UU workshop Deliberate field introduction of GMO (2005)
- NIOO Current Themes in Ecology "Influenza Ecology and Pandemics (2005)
- NWO Bessensap (+oral) (2006)
- WUR workshop human pathogens in the vegetable production chain (+oral) (2006)
- RIVM Workshop STEC (2007)
- WUR/FIMM symposium "Smeulende Vuurtjes" (+oral) (2007)

International Symposia, Workshops and Conferences (7 credits)

- 10th International Symposium on Microbial Ecology, Cancun, Mexico (+oral) (2004)
- 11th International Symposium on Microbial Ecology, Vienna, Austria (2007)
- 6th International Symposium on Shiga-toxin Producing E. coli infections, Melbourne, Australia (2007)

Teaching Activities

- Introduction to organic farming, Biologische landbouwsystemen
- Analyse van bedrijfssystemen, Bedrijfseconomie