Behaviour of *Escherichia coli* O157:H7 and *Salmonella enterica* during greenhouse butterhead lettuce production

Inge Van der Linden

Data, data, data. One cannot make bricks without clay.

Sherlock Holmes

Promotors: Prof. dr. ir. Mieke Uyttendaele
Department of Food Safety and Food Quality
Ghent University

Prof. dr. Marc Heyndrickx
Institute for Agricultural and Fisheries Research (ILVO)
Technology & Food Science Unit – Food Safety
&
Department of Pathology, Bacteriology and Poultry Diseases
Ghent University

Prof. dr. Martine Maes
Institute for Agricultural and Fisheries Research (ILVO)
Plant Sciences Unit - Crop Protection
&
Department of Biochemistry and Microbiology
Ghent University

Dean: Prof. dr. ir. Guido Van Huylenbroeck

Rector: Prof. dr. Anne De Paepe

Inge Van der Linden

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Gedrag van *Escherichia coli* O157:H7 en *Salmonella enterica* tijdens de serreteelt van botersla.

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Inge

2014

Table of Contents

List of Abbreviations

ANOVA	analysis of variance
Aw	water activity
BHI	brain heart infusion
BOD	biological oxygen demand
bp	base pair
BPW	buffered peptone water
CDC	Centers for Disease Control and Prevention
CFU	colony forming units
COD	chemical oxygen demand
Ct	cycle threshold
CT-SMAC	cefixime tellurite sorbitol MacConkey agar
DAEC	diffusely adherent *E. coli*
DEC	diarrhoeagenic *E. coli*
DNA	deoxyribonucleic acid
dpi	days post inoculation
EAEC	enteroaggregative *E. coli*
EFSA	European Food Safety Authority
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EMA	ethidium monoazide
EPEC	enteropathogenic *E. coli*
EPPO	European and Mediterranean Plant Protection Organization
ETEC	enterotoxigenic *E. coli*
EU	European Union
ExPEC	extraintestinal *E. coli*
FAO	food of animal origin
FAVV	Federaal Agentschap voor de veiligheid van de voedselketen
FoNAO	food of non-animal origin
GFP	green fluorescent protein
GW	groundwater
HUS	hemolytic uremic syndrome
IKKB	Integraal Keten Kwaliteit Beheersysteem

ILVO	Instituut voor Landbouw- en Visserijonderzoek
JA	jasmonic acid
LB	Luria Bertoni broth
LBA	Luria Bertoni agar
LFMFP	Laboratory of Food Microbiology and Food Preservation
log	logarithm (base 10)
M9	minimal medium
M-cells	microfold cells
mRNA	messenger RNA
NB	nutrient broth
NCTC	National Collection of Type Cultures
NMEC	neonatal meningitis *E. coli*
OD	optical density
OMP	outer membrane protein
PBS	phosphate buffered saline
PCA	plate count agar
PCR	polymerase chain reaction
PMA	propidium monoazide
PW	pond water
qPCR	quantitative PCR
RH	relative humidity
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SA	salicylic acid
SD	standard deviation
SDW	sterile distilled water
STEC O157	Shiga toxin producing *Escherichia coli* O157
sv.	serovar
T3SS	type III secretion system
TAL	top agar layer
TSA	tryptic soy agar/tryptone soy agar
TSB	tryptic soy broth/tryptone soy broth
UGent	Ghent University

UPEC	uropathogenic *E. coli*
USA	United States of America
VBNC	viable but not culturable
VTEC	Verotoxigenic *E. coli*
WPCA	water plate count agar
XLD	xylose lysine desoxycholate

Problem statement and outline

Fruits and vegetables, together called fresh produce, represent an important part of the human diet. In recent years, the fresh produce market has experienced rapid growth (Sapers *et al.* 2006; Warriner *et al.* 2009). Consumers want to lead a healthy lifestyle and this is encouraged by many governmental health agency campaigns which recommend to consume at least five daily servings of fruit and vegetables (Abadias *et al.* 2008; Van Boxstael *et al.* 2012). However, since the mid 1990's foodborne illness outbreaks linked to consumption of fresh fruits and vegetables were documented. Such outbreaks can be caused by different microorganisms such as *Listeria monocytogenes*, *Shigella flexneri*, *Campylobacter jejuni*, and viruses (hepatitis A and noroviruses), but most identified outbreaks were caused by *Salmonella* and *Escherichia coli* O157:H7 (*E. coli* O157:H7). Most of these are enteric bacterial pathogens which are traditionally associated with food products of animal origin like meat, dairy and eggs. A variety of fresh produce were implicated in outbreak reports: e.g. cantaloupe, tomatoes, alfalfa, lettuce, peppers, spinach, raspberry and radish sprouts (Aruscavage *et al.* 2006). Most outbreaks mainly occurred in the USA but also in other countries (Aruscavage *et al.* 2006). In the European Union (EU) foodborne verified outbreaks were linked to the consumption of vegetables, fruits, berries, juices (and products thereof) (EFSA and ECDC 2012; Van Boxstael *et al.* 2012). The impact of such outbreaks can be tremendous and the number of affected persons very high. This was illustrated with the German outbreak in 2011 which outnumbered the previous ones in both size and severity. The outbreak could, in all probability, be traced back to the consumption of fenugreek sprouts contaminated with *E. coli* O104:H4 grown from seeds imported from Egypt (EFSA and ECDC 2011) and had dramatic consequences. More than 50 people died and more than 4000 were sickened, of whom 852 developed the kidney-damaging complication called hemolytic uremic syndrome (HUS) (Mellmann *et al.* 2011). Besides the very severe consequences for public health also a significant economic impact was recorded. Spain, which was first incorrectly linked to the outbreak, has claimed losses from at least 51 million euro caused by produce withdrawal and nearly 200 million euro due to production loss. In addition, all the health care expenditure (such as diagnostics, epidemiological analyses, hospitalization, dialysis, future renal organ transplantations) must be included as long-term social expenses (Karch *et al.* 2012).

Different causes can be assigned for the occurrence of such large-scale outbreaks including increased consumption of fresh produce, changes in farming practices such as centralized production and most importantly the lack of an inactivation step of the pathogen during production as fresh produce are consumed raw. Even if decontamination of produce is attempted by e.g. washing with a hypochlorite solution, research shows that these techniques are often not successful in removing the pathogens completely (Gomez-Lopez *et al.* 2008). The understanding of the

microbiology of fresh produce and the routes of infection is, therefore, the first essential step to effectively elaborate control measures.

This PhD research studies the survival, attachment and gene expression of the enteric pathogens *E. coli* O157:H7 and *Salmonella enterica* during butterhead lettuce production. The pathogens *E. coli* O157:H7 and *Salmonella* were selected because of their strong association with produce-related outbreaks (114). Lettuce was taken as model plant for leafy greens which are frequently implicated in outbreaks (Rangel *et al.* 2005). More specifically butterhead lettuce (*Lactuca sativa* L. var. *capitata*) was chosen because of its economic value for Belgium and the lack of knowledge about this typical northern European lettuce crop type regarding contamination with human pathogens. The focus lies on contamination of the crop before harvest, also called preharvest contamination. This has been less intensively studied in comparison with postharvest contamination. Furthermore, it is important to be able to control the food safety risk in the preharvest stage as contamination at this stage may spread over a large volume of fresh produce in the postharvest stage during washing, mixing of the produce (in fresh-cut processing) or packing (Whipps *et al.* 2008). Finally, to better understand a natural contamination event, not only the behavior of freshly cultured, unstressed pathogens but also the behavior of stressed pathogens was investigated. The PhD-study was situated within the SALCOSLA project. This project was funded by the Belgian Federal Public Service Health, Food Chain Safety and Environment in the period April 2009 to April 2013 in order to gain more information on the risk factors for introduction and persistence of enteric bacterial pathogens during the Belgian primary production of leafy greens.

This thesis starts with a general introduction (**chapter 1**), which contains a short description of the outbreaks linked to fresh produce, the pathogens *E. coli* O157:H7 and *Salmonella*, the model plant butterhead lettuce and the transmission of the pathogens to the vegetable production chain. Furthermore, a schematic overview of the existing research about contamination of lettuce with enteric pathogens is presented which is used to show where the present PhD research tries to contribute to fill the knowledge gaps. In **chapter 2**, the survival characteristics of *E. coli* O157:H7 and *Salmonella* on lettuce seeds and seedlings are investigated. The aim of this chapter was to check whether contaminated seeds could be a potential contamination source in greenhouse cultivation. This chapter shows that both *E. coli* O157:H7 and *Salmonella* were able to proliferate on lettuce seedlings even after residing for a period of two years on the lettuce seeds. The survival of both pathogens introduced by irrigation water on young and nearly mature butterhead lettuce plants is studied in **chapter 3**. Both plant factors, such as crop stage, and cultivation factors, such as temperature, relative humidity, and irrigation treatment were investigated. These experiments were

conducted with freshly cultured pathogens suspended in a sterile buffer. But in a natural contamination event this is, however, unlikely to occur. The pathogens may behave differently in case of prior residence in the complex matrix of irrigation water before being transferred to the plant leaves. **Chapter 4** combines, therefore, research on the survival of the pathogens in five different irrigation water samples from Belgian lettuce production sites and the influence of the inoculum carrier on the survival of the pathogen on lettuce. Subsequently, the effect of the residing time in irrigation water on the attachment of the pathogens on lettuce is described in **chapter 5. Chapter 6** goes back to the basics. As enteric pathogens on plants are in a completely different environment than in the animal bowel, the pathogen needs to use different strategies to survive in this specific environment. Whole genome transcriptional profiles were generated from *E. coli* O157:H7 cells inoculated on the leaves of growing butterhead lettuce to reveal which genetic underlying mechanisms are necessary for *E. coli* O157:H7's survival on plants.

Finally, the major findings of this work are summarized and the future research perspectives are discussed in **chapter 7**.

Chapter 1:

General introduction

1.1 Foodborne illness

Foodborne illness or foodborne disease is defined as any illness that results from the consumption of contaminated foods. Contamination of food can be caused by the presence of pathogenic bacteria, viruses or parasites or due to the presence of chemicals or natural toxins. This thesis focusses on pathogenic bacteria that are together with viruses the dominant cause of foodborne illness (Anonymous, 2013a).

Traditionally, foodborne outbreaks had a local character, people became ill after e.g. a wedding reception where contamination of the food occurred shortly before consumption. This kind of contamination can usually be attributed to a food handling error such as improper heating or interruption of the cold chain. Nowadays, however, large scale outbreaks occur more frequently. These outbreaks are often diffuse and widespread, involving many countries, states, even on different continents (Tauxe 1997). The root cause is probably the change in the way food is produced and distributed nowadays. Throughout the years, food production went through a process of intensification and centralization whereas the distribution was globalized. Furthermore, a higher proportion of the population is susceptible for foodborne illness since the number of susceptible people such as elderly and immunocompromized persons, is increasing. Hotspots of foodborne pathogen emergence are concentrated in regions with a high human population density and human population growth such as South and Southeast Asia, but also Europe and North America (Figure 1.1) (Jones *et al.* 2008).

Most of the foodborne pathogenic bacteria are enteric pathogens, causing gastrointestinal diseases. Furthermore, the bacteria are zoonotic pathogens which means that they have an animal reservoir from which they spread to humans, sometimes by a vector (Zambrana-Torrelio *et al.* 2012). As a consequence, most of the foodborne outbreaks are caused by animal derived products such as meat, eggs and dairy. Since 20 years, however, the pathogens have also been detected in new food vehicles such as plant derivatives and fresh produce (Tauxe 1997). One of the first evidences for the fact that fresh produce could be contaminated with zoonotic enteric pathogens occurred in 1996. 10 000 Japanese people, the majority being school-age children, were infected with *E. coli* O157:H7 after the consumption of contaminated radish sprouts (Watanabe *et al.* 1999). Since then, more large spread outbreaks with enteric bacterial pathogens on fresh produce were reported. The majority of the reported outbreaks occurred in the USA, but similar trends towards an increased health risk related to the consumption of fresh produce can be seen in Europe. In the following two paragraphs, the foodborne outbreak statistics from the USA and Europe will be discussed in some further detail.

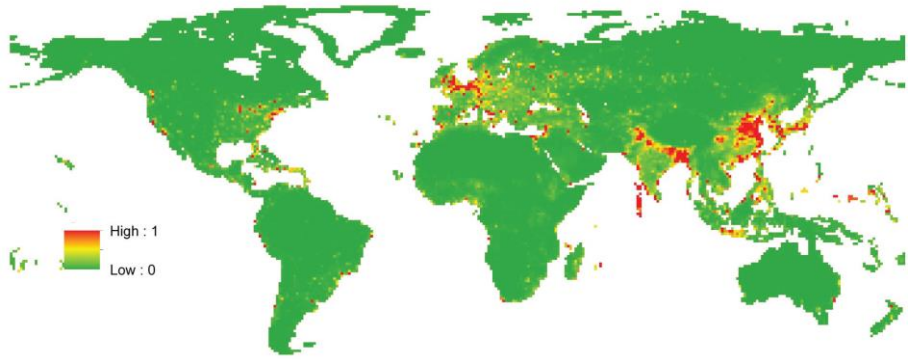


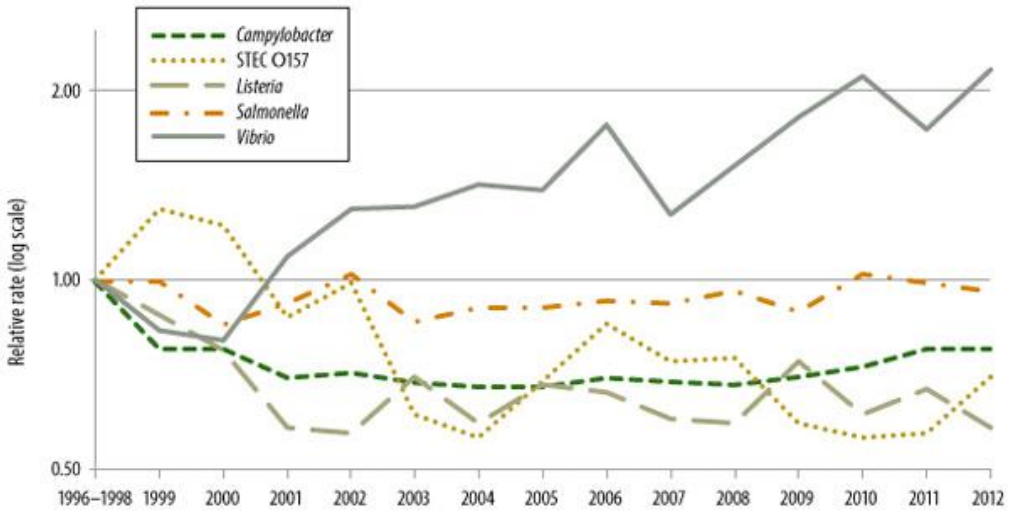
Figure 1.1: Relative risk of foodborne emerging infectious disease events, based on Jones *et al.* (2008). Human population density and human population growth, were the most important variables.

1.2 Outbreaks linked to fresh produce

1.2.1 USA

In the USA, data on foodborne outbreaks are collected by the state, local and territorial public health agencies. These data are reported on a voluntary basis to the Foodborne Disease Outbreak Surveillance System and are analyzed and reported by the Centers for Disease Control and Prevention (CDC).

An analysis based on the data that were reported by the CDC between 2000 and 2008 estimated that each year, 31 known foodborne pathogens caused between 6.6-12.7 million illnesses which lead to 40 000-76 000 hospitalizations and 712-2268 deaths. The majority of illnesses in this period was caused by viruses (59%), dominantly norovirus. Although illness caused by viruses often occurs, the illness is in general less severe. This can be deduced from the hospitalization rate and death rate which were respectively 27% and 12%. Parasites (mainly *Toxoplasma gondii*), on the opposite, were responsible for only 2% of illnesses, but 25% of deaths. But the highest number of deaths and hospitalizations (64% each) were caused by bacteria although they were responsible for only 39% of illnesses. The most important bacterial pathogens that caused hospitalization were nontyphoidal *Salmonella* spp. (35%) (see also 1.3.2) and *Campylobacter* spp. (15%). Nontyphoidal *Salmonella* spp. was also responsible for the highest number of deaths (28%), followed by *Listeria monocytogenes* (19%). The incidence of *E. coli* O157:H7 was much lower (3.8% of the hospitalizations, 1.5% of the deaths). This low incidence of *E. coli* O157:H7 infection is the result of improvements in sanitation at slaughter and meat processing plants which were implemented between 1996-1998. Since then a decrease with 30-40% of *E. coli* O157:H7 infections could be seen. Since 2003, however, there has only been little progress (CDC 2011f). Similar trends were seen for *Campylobacter* and *Listeria* infections. This is in contrast with *Salmonella* infections which are not decreasing (Figure 1.2).



* Shiga toxin-producing *Escherichia coli*.
† The position of each line indicates the relative change in the incidence of that pathogen compared with 1996–1998. The actual incidences of these infections cannot be determined from this graph. Data for 2012 are preliminary.

Figure 1.2: Relative rates of laboratory-confirmed infections with *Campylobacter*, Shiga toxin producing *E. coli* O157:H7 (STEC O157), *Listeria*, *Salmonella* and *Vibrio* compared with 1996-1998 rates, by year. – Foodborne Diseases Active surveillance Network; United States, 1996-2012 (CDC 2012)

Contaminated meat was the main cause of foodborne outbreaks caused by *Salmonella* (36%) and pathogenic *E. coli* (46%) between 1996 and 2005. In the same period fresh produce were the second most important cause of foodborne outbreaks (16% of the registered outbreaks for *Salmonella* and 27% for pathogenic *E. coli*). Comparative risk assessment based on the analysis of the outbreaks with fresh produce that occurred in the USA from 1996-2008 indicates that the highest risk is presented for leafy greens and *E. coli* O157:H7 (EHEC) followed by *S. enterica* and tomatoes and *S. enterica* and leafy greens (Anderson *et al.* 2011). This assessment took nine criteria into consideration regarding both health impact and consequences of particular pathogens as well as factors related to dose-response relationships, consumption, prevalence of the hazards and the possibility of growth of the hazards during shelf life (Anderson *et al.* 2011; EFSA and BIOHAZ 2012).

The first large-scale outbreak in the USA with fresh produce occurred in 2005 and 2006. More than 450 people from the USA and Canada became ill after the consumption of contaminated tomatoes (Bidol *et al.* 2007). The outbreak strain was isolated from the pond water used to irrigate the tomato fields (Greene *et al.* 2008). In the same period, a large outbreak occurred with *E. coli* O157:H7 on spinach with almost 200 illnesses and 3 fatalities (CDC 2006a). *E. coli* O157:H7 isolates which were isolated from spinach, were also found in wild pig feces, the feces of several cows, and in a stream of one of the four spinach farms in the area (Warnert 2007). A third large outbreak with *Salmonella* occurred two years later, this outbreak was initially thought to be associated with tomatoes but later linked to peppers (Barton Behravesh *et al.* 2011). All these outbreaks were typically multistate or

multinational outbreaks. An overview of the most important fresh produce related outbreaks with enteric bacterial pathogens in the USA is given in Table 1.1.

Table 1.1: Enteric bacterial pathogen outbreaks linked to fresh produce from 2005-2011 redrafted from Olaimat and Holley 2012.

Location	Year	Pathogen	Produce	Human cases (deaths)	References
USA, Canada	2005	*Salmonella*	Tomatoes	459	(CDC 2007)
USA	2006	*E. coli* O157:H7	Spinach	199 (3)	(CDC 2006c)
USA, Canada	2006	*Salmonella*	Fruit salad	41	(Landry *et al.* 2007)
USA	2006	*Salmonella*	Tomatoes	183	(CDC 2006b)
USA	2006	*E. coli* O157:H7	Lettuce	81	(FDA 2007)
USA	2006	*E. coli* O157:H7	Spinach	22	(Grant *et al.* 2008)
North America, Europe	2007	*Salmonella*	Basil	51	(Pezzoli *et al.* 2007)
USA, Canada	2008	*Salmonella*	Peppers	1442 (2)	(CDC 2008b; Mody *et al.* 2011)
USA, Canada	2008	*E. coli* O157:H7	Lettuce	134	(Warriner and Namvar 2010)
USA	2008	*Salmonella*	Cantaloupe	51	(CDC 2008a)
USA, Canada	2008	*Salmonella*	Peanut butter	714 (9)	(CDC 2009b)
USA	2009	*Salmonella*	Alfalfa sprouts	235	(CDC 2009a)
USA	2010	*E. coli* O145	Lettuce	26	(CDC 2010a)
USA	2010	*Salmonella*	Alfalfa sprouts	44	(CDC 2010b)
USA	2010	*L. monocytogenes*	Fresh cut produce (celery)	10 (5)	(FDA 2010)
USA	2011	*Salmonella*	Alfalfa and mixed sprouts	140	(CDC 2011b)
USA	2011	*Salmonella*	Cantaloupe	20	(CDC 2011c)
USA	2011	*Salmonella*	Papaya	106	(CDC 2011d)
USA	2011	*L. monocytogenes*	Cantaloupe	146 (31)	(CDC 2011e)
USA	2011	*E. coli* O157:H7	Strawberries	15 (1)	(FDA 2011)
USA	2011	*E. coli* O157:H7	Lettuce	60	(CDC 2011a)

1.2.2 European Union

In the European Union, the annual reporting of zoonosis (including foodborne outbreaks), zoonotic agents and antimicrobial resistance present in animal populations and in the food chain is mandatory (Directive 2003/99/EC). Data has to be collected for *Salmonella* spp., thermotolerant *Campylobacter* spp., *Listeria monocytogenes*, Shiga toxin producing *Escherichia coli*, *Mycobacterium bovis*, *Brucella*

spp., *Trichinella* spp. and *Echinococcus* spp.. Analyzing and summarizing the collected data and annual reporting is coordinated by the European Food Safety Authority (EFSA). It has, however, to be noted that the foodborne outbreak investigation systems at the national level are not harmonized between the 27 Member States.

Data from EFSA, based on the reported foodborne outbreaks by EU countries, showed that in the European Union in 2011, campylobacteriosis was the most commonly reported zoonosis with 220 209 cases. *Salmonella* caused the second most infections (95 548 cases) and caused the highest number of reported foodborne outbreaks. But the number of *Salmonella* infections is steadily decreasing. It is assumed that this reduction is a result of the successful *Salmonella* control programs in poultry populations. In food, *Salmonella* is most often detected in meat and meat derivatives, mainly in broiler meat (5.9% of the samples) and pig meat (0.7% of the samples), and was present in a very low proportion of table eggs (0.1%). The two most common serovars were *Salmonella* Typhimurium and Enteritidis. (EFSA 2013)

Similar to the USA, the number of Shiga toxin producing *E. coli* (STEC) cases was much lower. In 2011, 9485 confirmed STEC cases were reported. This was almost twice as much in comparison with earlier years and was a result of the large *E. coli* O104:H4 outbreak. *E. coli* O157:H7 is most often detected in fresh bovine meat (0.3%). (EFSA 2013)

EFSA makes a clear distinction between foodborne outbreaks related to food of animal origin (FAO) or food of non-animal origin (FoNAO). The definition of the latter is quite broad and includes '*all food derived from plants which are ubiquitous in their distribution providing a major component of almost all meals*'. This range of foods and food components comprises a wide range of fruit, vegetables, salads, seeds, nuts, cereals, herbs, spices fungi and algae, which are commonly consumed in a variety of forms. Between 2007-2011, 19 EU countries reported foodborne outbreaks with strong evidence that food of non-animal origin was implicated. From 2007-2010 8% of the outbreaks, 16% of the cases, 5% of the hospitalizations and 6% of the deaths were caused by outbreaks related to FoNAO. When the data from 2011 are taken into consideration, the percentages increase dramatically to 10% of the outbreaks, 26% of the cases, 35% of the hospitalizations and 46% of the deaths. These high values are associated with the *E. coli* O104:H4 outbreak in Germany which is believed to be associated with sprouted seed consumption. (EFSA and BIOHAZ 2012)

The highest number of foodborne outbreaks related to FoNAO was reported for the combination norovirus and raspberries followed by norovirus and leafy greens eaten raw as salads. The remaining most frequently reported combinations were: *Salmonella* spp. and sprouted seeds, *Salmonella* spp. and leafy greens eaten raw as salads, *Bacillus* spp. and spices and dry herbs, *Shigella* spp. and fresh

pods, legumes or grain, STEC and sprouted seeds and norovirus and bulb or stem vegetables (Table 1.2) (EFSA and BIOHAZ 2012). When the same risk ranking tool as described for USA was used for the EU data from 2007-2011, *Salmonella* combined with leafy greens eaten raw as salads was considered to have the highest risk, followed by *Salmonella* on bulb and stem vegetables, tomatoes and melons and pathogenic *E. coli* on fresh pods, legumes and grain (Anderson *et al.* 2011). So for both USA and the EU, the assessment points towards leafy greens as produce related with the highest bacterial pathogen risk. Based on these data, this thesis focusses on pathogenic *E. coli*, more specifically *E. coli* O157:H7, and *Salmonella* and leafy greens, with lettuce as model plant. These organisms will be discussed in the following sections.

1.3 Characteristics of the investigated pathogens

Escherichia coli O157:H7 and *Salmonella* are Gram-negative bacteria of the Enterobacteriaceae family. They are closely related to other important foodborne pathogens such as *Yersinia enterocolitica*, *Shigella* spp. and *Cronobacter* spp., and to plant pathogens such as *Erwinia* spp., *Pantoea* spp. and *Pectobacterium* spp. which may cause blights, wilts, and soft rots (Butela and Lawrence 2010; Fletcher *et al.* 2013). *Salmonella* and *Escherichia* are quite closely related, the two genera show about 50% genomic hybridization and probably diverged from a common ancestor 120-140 million years ago (Madigan *et al.* 2012).

Both pathogens are similarly transmitted via the fecal-oral pathway. The bacteria are directly or indirectly ingested via fecal material. Subsequently, both species need to survive the very low pH of the stomach before entering the intestinal tract. From this point *Salmonella* preferably colonize the small intestine whereas *E. coli* O157:H7 colonization is located in the large intestine.

1.3.1 *Escherichia coli* O157:H7

1.3.1.1 Taxonomy, characteristics and E. coli pathovars

Escherichia coli (*E. coli*) is a Gram-negative, non-sporulating, facultative anaerobe bacillus and a member of the family of Enterobacteriaceae. Within this family, the bacterium belongs to the coliforms as it is able to ferment lactose rapidly while producing acid and gas. Since the early 1940's, *E. coli* isolates are classified by serotype as defined by Kaufmann (Achtman and Pluschke 1986). Following this system, different *E. coli* strains can be identified based on the presence of O-antigens which are lipopolysaccharides, H-antigens which are flagellar antigens and sometimes the capsular antigen K. *E. coli* strains with the same O-antigen form serogroups, whereas *E. coli* strains which share the same O and H-antigens belong to the same serotype.

Table 1.2 Number of outbreaks reported for most frequent combinations of foodborne pathogen and Food of non-animal origin (2007-2011) following EFSA (EFSA and BIOHAZ 2012).

Foodstuff implicated(c)	Causative agent	Number of outbreaks	Human cases	Number of cases hospitalized	Deaths	Number of reporting countries
Raspberries	Norovirus	27	913	3	0	3
Leafy greens eaten raw as salads	Norovirus	24	657	1	0	3
Sprouted seeds	*Salmonella* spp.	11	521	76	1	8
Leafy greens eaten raw as salads	*Salmonella* spp.	7	438	29	0	5
Spices and dry herbs	*Bacillus* spp.	7	343	0	0	4
Fresh pods, legumes and grain	*Shigella* spp.	4	268	3	0	3
Sprouted seeds	STEC	3	3 830	2 381	53	3
Bulb and stem vegetables	Norovirus	2	18	0	0	2

(a) This table lists all food type and pathogen group combinations for which more than one outbreak assigned to one single product was reported between 2007-2011 with the aggregated numbers of human cases, hospitalizations and deaths. Combinations of pathogen and FoNAO type were ranked by the number of outbreaks reported. When the same number of outbreaks was reported for more than one combination of pathogen and food type, these combinations were ranked by the number of human cases.
FoNAO, which may include one or more cooked ingredients (e.g. cooked vegetable salads), (ii) foods which normally are subjected to a processing step which should inactivate vegetative cells (e.g. rice, pasta and cereals), (iii) other processed FoNAO, (iv) non-specified fruit or (v) outbreaks where no detailed information was available to be able to identify the specific implicated FoNAO were excluded.

E. coli is the most predominant aerobic organism in the gastrointestinal tract of warm-blooded animals and reptiles (Tenaillon *et al.* 2010). They are mainly located in the large intestine, especially in the caecum and the colon where they reside in the mucus layer that covers the epithelium. The bacterium is highly specialized in metabolizing mucus-derived sugars such as gluconate and it is excreted in the feces together with the degraded mucus. In humans the concentration per gram of feces varies from 10^7 to 10^9 colony forming units (CFU) while in domestic animals this is lower (up to10^6 CFU/g feces). *E. coli* is, therefore, often used as an indicator of fecal pollution (Baylis *et al.* 2011). But apart from being a widespread gut commensal, certain isolates have been shown to be pathogenic in either animals or humans worldwide. The pathogenic strains have been associated with gastrointestinal diseases (diarrhoeagenic *E. coli*) or extraintestial diseases and have been categorized into different pathogenicity groups (pathovars), based on their virulence properties, pathogenesis and clinical manifestations. Eight pathovars and their mechanisms of diseases are well known and are described in Table 1.3 (Croxen and Finlay 2009).

This thesis focuses on one of these pathovars, enterohemorrhagic *E. coli* (EHEC), and more specifically on serotype *E. coli* O157:H7 which is the most common member of the EHEC group. The first outbreak caused by *E. coli* O157:H7 occurred in Oregon and Michigan (USA) in 1982 and was related to the consumption of hamburgers in a restaurant chain. In Belgium, the first registered outbreak occurred five years later in 1987 (Pennington 2010). Since 1993, after a large multistate *E. coli* O157:H7 outbreak was linked to undercooked ground beef patties sold from a fast food restaurant chain, *E. coli* O157:H7 became broadly recognized as an important human pathogen (Behravesh *et al.* 2012)

Apart from *E. coli* O157:H7 four other typical EHEC serotypes exists, namely O26:[H11], O103:H2, O111:[H8] and O145:[H28]. Typical EHEC strains produce one or two Shiga toxin types and harbor a genomic island called the locus of enterocyte effacement (LEE) (Beutin *et al.* 2009).
People infected with EHEC suffer from bloody diarrhea (hemorrhagic colitis) and 10-15% of the patients develop hemolytic uremic syndrome (HUS) 5-13 days after the onset of diarrhea (Pennington 2010). HUS leads to destruction of red blood cells, acute kidney failure, and a low platelet count and is potentially fatal especially for the young, elderly and immunocompromized. It is the major cause of acute renal failure in children under five years of age (Caprioli *et al.* 2005). In EU in 2009, 63.2% of the reported HUS cases could be assigned to this age category (EFSA 2011a).

Table 1.3: Overview of the eight pathogenic *E. coli* pathovars following Croxen and Finlay (2009) *(Croxen and Finlay 2009).*

	Pathovar	Colonization site in the human body	Illness
Diarrhoeagenic *E. coli* (DEC)	enteropathogenic *E. coli* (EPEC)	small bowel	diarrhea in infants in developing countries
	enterohemorrhagic *E. coli* (EHEC)	**large bowel**	**severe gastroenteritis in developed countries**
	enterotoxigenic *E. coli* (ETEC)	small bowel	travelers' diarrhea
	enteroinvasive *E. coli* (including *Shigella*) (EIEC)	large bowel	watery diarrhea
	enteroaggregative *E. coli* (EAEC)	small bowel, large bowel	persistent watery and mucoid diarrhea, travelers' diarrhea
	diffusely adherent *E. coli* (DAEC)	small bowel	
Extraintestinal *E. coli* (ExPEC)	uropathogenic *E. coli* (UPEC)	bladder, kidney, bloodstream	urinary tract infections
	neonatal meningitis *E. coli* (NMEC)	bloodstream, brain	neonatal meningitis

1.3.1.2 Virulence characteristics

Infection with *E. coli* O157:H7 starts with ingestion of the pathogen. In the colon, *E. coli* O157:H7 can attach to hosts intestinal absorptive epithelial cells (enterocytes) by means of fimbrial adhesins, which are cellular appendages that are thinner and shorter than flagella. This triggers the expression of several virulence genes, grouped together on the locus of enterocyte effacement (LEE) and induces the secretion of proteins (effectors) into the cell plasma of the enterocytes through a needle like structure, a type III secretion system (T3SS). One of the secreted proteins is the translocated intimin receptor (Tir), this protein becomes integrated on the cell surface of the epithelial cell and serves as a binding place to a protein in the bacterial cell wall called intimin (Eae). Binding results in a cascade of signaling (a.o. mitochondrial-associated protein (Map), and EspF, EspG) and eventually in the destruction of the microvilli's brush border of the enterocyte through the formation of a pedestal and in a tight connection between pathogen and the host cell and deregulation of ion exchangers (Caprioli *et al.* 2005; Croxen and Finlay 2009). This combination leads to diarrhea as the destruction of the microvilli's brush border leads to a decrease in water absorption. Furthermore, the effectors secreted by the T3SS can affect the ion exchangers such as Cl^-OH^- (EspG) and Na^+OH^+ (EspF) and misloc alize the membrane water channels (aquaporins). A combination of these effectors (EspF, Map, Tir) also inhibit another major waterpump (sodium-D-glucose cotransporter 1 (SLGT1)) and a serotonin transporter involved in uptake of serotonin which needed for intestinal absorption and secretion of electrolytes an fluids (Croxen and Finlay 2009; Holmes *et al.* 2010).

The key factor in the development of HUS is the production of Shiga toxins (Stx). These toxins,

sometimes also called Vero toxins (Vtx), inhibit protein synthesis within eukaryotic cells. Pathogenic *E. coli* which are able to produce these toxins are, therefore, also called Stx producing *E. coli* (STEC) or Vtx producing or Verotoxigenic *E. coli* (VTEC). *E. coli* O157:H7 can produce two different Shiga toxins. Stx1 is very similar to the type 1 toxin produced by *Shigella dysenteriae* (this also explains the name Shiga toxins) whereas Stx2 is genetically and immunologically distinct, has different variants and is more prevalent in hemorrhagic colitis and HUS than Stx1 (Caprioli *et al.* 2005; Croxen and Finlay 2009; Pennington 2010).

The Shiga toxins are not released by a specific secretory mechanism. The toxin genes are encoded on bacteriophages, or remnants thereof, which are lysogenized in the bacterial chromosome (Verstraete 2012). The toxins are released when bacteriophage mediated bacteriolysis occurs in response to DNA damage and the SOS response. This is also the reason why treatment with antibiotics is not recommended as this may lead to an increased release of the toxins. Shiga toxins consist of an A- and B-unit. The B-unit can bind on glycolipid receptors Gb3 that are e.g. present on cells in het human intestinal mucosa and the surface of kidney epithelial cells. Binding enables the holoenzyme to enter the cytosol and the A-subunit is transported to the cytosol where it is activated by proteolytic cleavage. This activated subunit, cleaves off on its turn, a single adenine residue from the 28S rRNA of the 60S ribosomal subunit of the host. This results in the inhibition of protein synthesis which induces a ribotoxic stress response that can lead to programmed (apoptosis) or unprogrammed (necrosis) cell death of the infected cell (Caprioli *et al.* 2005; Croxen and Finlay 2009; Pennington 2010; Chekabab *et al.* 2013).

Furthermore, *E. coli* O157:H7 isolates are also characterized by the presence of a virulence plasmid pO157 which encodes several other virulence factors. The biological role of pO157 is, however, not yet fully understood, but has a role in biofilm development (Lim *et al.* 2010; Puttamreddy *et al.* 2010). A schematic overview of an *E. coli* O157:H7 infection is presented in Figure 1.4.

1.3.2 *Salmonella*

1.3.2.1 Taxonomy, characteristics

Salmonella covers a diverse range of bacteria that cause a spectrum of diseases in many hosts (Mastroeni and Maskell 2006). The first laboratory confirmed outbreak caused in human occurred in 1888 when 58 people became ill after the consumption of meat from an emergency slaughtered cow. The bacterium was isolated from both the consumed meat and the organs of a man for whom the infection was fatal (Bell and Kyriakides 2008). The *Salmonella* genus is divided in two species: *Salmonella* bongori and *Salmonella* enterica, which on its turn is divided into 7 subspecies, each with

different serovars. A *Salmonella* serovar is typically (but not always) named after the place of origin, such as *Salmonella enterica* subspecies *enterica* serovar Tennessee. Serovars names may be

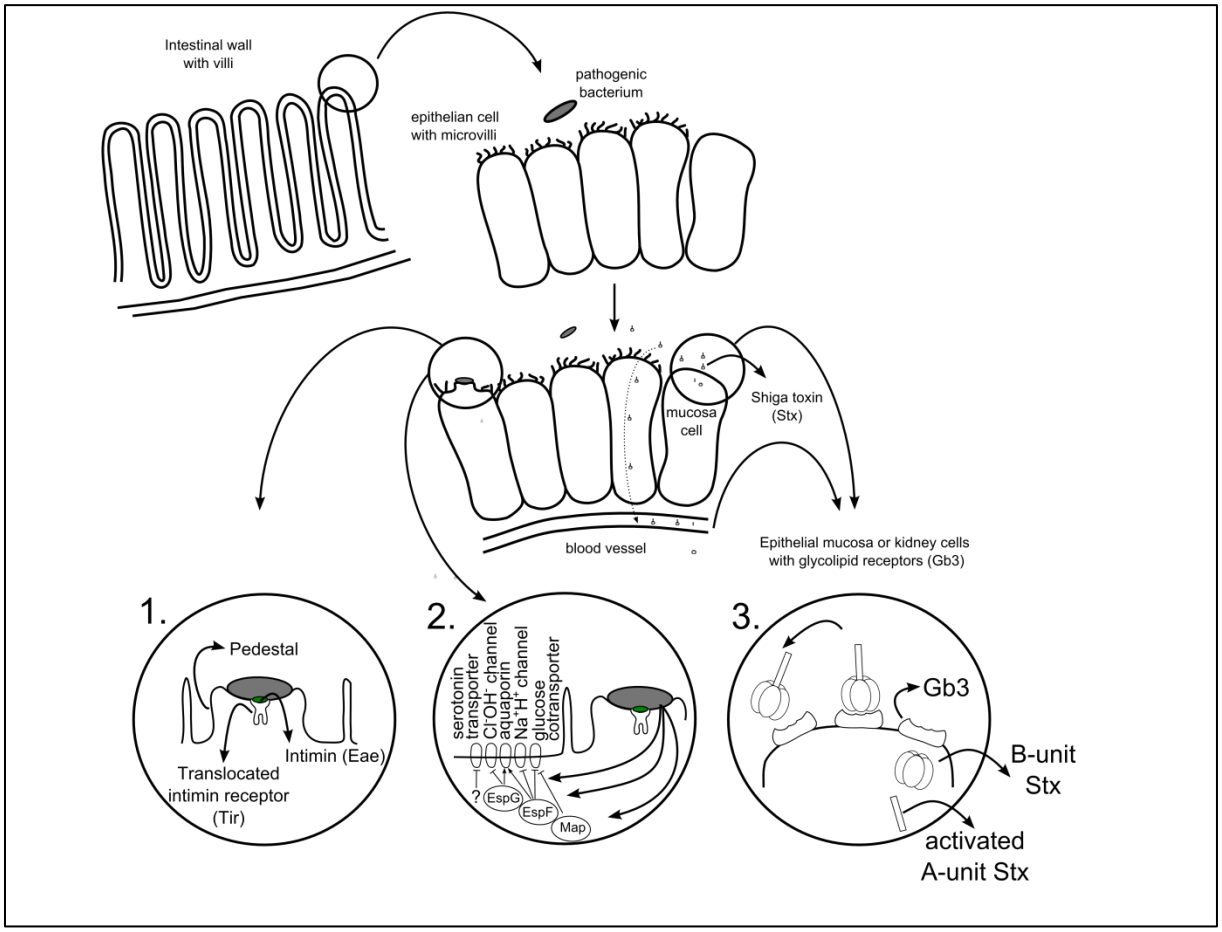


Figure 1.3: Virulence characteristics and pathogenesis of *E. coli* O157:H7. Binding of *E. coli* O157:H7 to an enterocyte/epithelial cell results in the formation of a pedestal and destruction of the microvilli's brush border (1) and in deregulation of a.o. the water channels and ion exchangers (2). Both events results in diarrhea. Released Shiga toxin binds on glycolipid receptors Gb3 that are e.g. present on cells in het human intestinal mucosa and the surface of kidney epithelial cells. In the cytosol, the A-subunit is This activated subunit, cleaves off a single adenine residue from the 28S rRNA of the 60S ribosomal subunit of the host. This results in the inhibition of protein synthesis which induces a ribotoxic stress response that can lead to programmed (apoptosis) or unprogrammed (necrosis) cell death of the infected cell (Caprioli *et al.* 2005; Croxen and Finlay 2009; Pennington 2010; Chekabab *et al.* 2013) (3). Figure partially redrafted and adjusted from (Caprioli *et al.* 2005; Croxen and Finlay 2009)

abbreviated, by convention, as the genus name and the serovar name, in this case *Salmonella* Tennessee. The most important subspecies is *Salmonella enterica* subsp. *enterica* (subspecies I) because it is responsible for 99% of human illness caused by *Salmonella* (Anjum *et al.* 2005; Lan *et al.* 2009). Two serovars, Typhi and Paratyphi, which are mainly found in developing countries cause enteric fever and has a high mortality rate (estimates of approximately 200 000 deaths per year) (Broz *et al.* 2012). Together they are called typhoid *Salmonella*. But most of the serovars cause salmonellosis, a disease characterized by diarrhea, fever, abdominal pain, nausea, and sometimes vomiting. These strains are also known as nontyphoid *Salmonella*. Nontyphoid infections are often mild, do not require antibiotics and last only a few days. However, in susceptible patients, the

infection may be more serious when the bacteria were able to enter the bloodstream or when dehydration caused by severe diarrhea becomes life threatening. Salmonellosis has also been associated with long term and sometimes chronic diseases such as reactive arthritis (Ohl and Miller 2001).

The reservoir of *Salmonella* is the intestinal tract of domestic and wild animals. The most commonly isolated serovar worldwide is serovar Typhimurium and his multidrug resistant DT104 clone which carries chromosomally based resistance to ampicillin, chloramphenicol, TMP-SMZ, streptomycin, and tetracycline. This is in contrast with Europe where serovar Enteritidis, often related with eggs/chicken meat outbreaks, is most frequently isolated (Lan *et al.* 2009; Schikora *et al.* 2011).

1.3.2.2 Virulence characteristics

Salmonella possesses several virulence genes in order to be able to cope with the defense strategies of the host. These genes are grouped in several large regions on the chromosome which are also called *Salmonella* pathogenicity islands. Up to eight pathogenicity islands are known and these are just like for *E. coli* O157:H7, probably horizontally acquired through bacteriophages (Broz *et al.* 2012).

Salmonella infection starts with ingestion of the pathogen. After passage through the acidic stomach, the remaining pathogens have to compete with the residential microbiota for nutrients and to traverse the mucus layer in which several antimicrobial peptides are present. By altering the structure of its membrane bound proteins, *Salmonella* can prevent the binding of those antimicrobials which mainly affect its cell wall integrity. Furthermore, *Salmonella* is also able to react with reactive oxygen species generated during inflammation, thereby forming a new respiratory electron acceptor. In this way, the pathogens turns the host defense into a growth advantage over the resident bacteria (Broz *et al.* 2012). To attach to the epithelium in the intestine, the pathogen uses cellular appendages such as fimbriae, the cell surface polysaccharide O-antigen, and the flagellar H-antigen (Madigan *et al.* 2012). *Salmonella* employs multiple strategies to establish virulence and pathogenesis, for which an initial prerequisite is adherence to the cells. Adherence requires a.o. several adhesins and fimbria. The pathogens mainly attach to the microfold cells (M-cells). These cells do not possess microvilli but microfolds and they have the function to transport antigens and bacteria to the non-intestine side and deliver them via transcytosis to cells of the immune system (such as macrophages, dendritic cells, lymphocytes) (Broz *et al.* 2012; Anonymous 2013b). On the M-cells, *Salmonella* is recognized by pattern-recognition receptors which recognize pathogen-associated molecular patterns (PAMP's) such as specific lipopolysaccharide (LPS) and flagellin. From this point, *Salmonella* is transported to the non-intestine side. Secondly, *Salmonella* can also attach to and actively invade other epithelium cells by injecting specific effector proteins into the cells by

means of a T3SS encoded by pathogenicity island I. Subsequently, effectors (a.o. SopE/SopE2, SopB and SipA) are injected and induce rearrangements of the enterocyte's cytoskeleton with the formation of membrane ruffles which enclose the bacterium and internalization as a result (endocytosis). Effectors of the first T3SS but mainly a second T3SS induce the transformation of the phagosome into a *Salmonella containing vacuole* (SCV) in which the bacterium can survive and replicate. Recently, it was discovered as well that *Salmonella* can be directly taken up by specific dendritic macrophages which are able to disrupt the tight junctions of the epithelial cells (Broz *et al.* 2012). On the non-intestine side, the *Salmonella* bacteria may be taken up by cells of the host's immune defense which normally destroys harmful elements and dead cells (macrophages). Here again, *Salmonella*, is able to enter and to form a vacuole where it is protected and can multiply by use of its second T3SS (Broz *et al.* 2012). The host receptors recognize a.o. these conserved T3SS molecules which leads to the expression and secretion of cytokines which amplify the inflammatory response with an acute, mucosal gut inflammation as a result (Broz *et al.* 2012). In the macrophages, a.o. pyroptosis, a kind of programmed cell death, is induced which force the protected pathogens to be exposed again to extracellular immune defenses. But from this point the pathogen may also disseminate through the blood stream accumulating in mesenteric lymph nodes and, ultimately, the spleen. Other virulence factors that *Salmonella* uses are a.o. the production of superoxide dismutase in order to quench reactive oxygen species produced by the host, the production of two siderophores, enterobactin and salmochelin, in response to iron deprivation (Ibarra and Steele-Mortimer 2009). A schematic overview of a *Salmonella* and *E. coli* O157:H7 infection is presented in Figure 1.4.

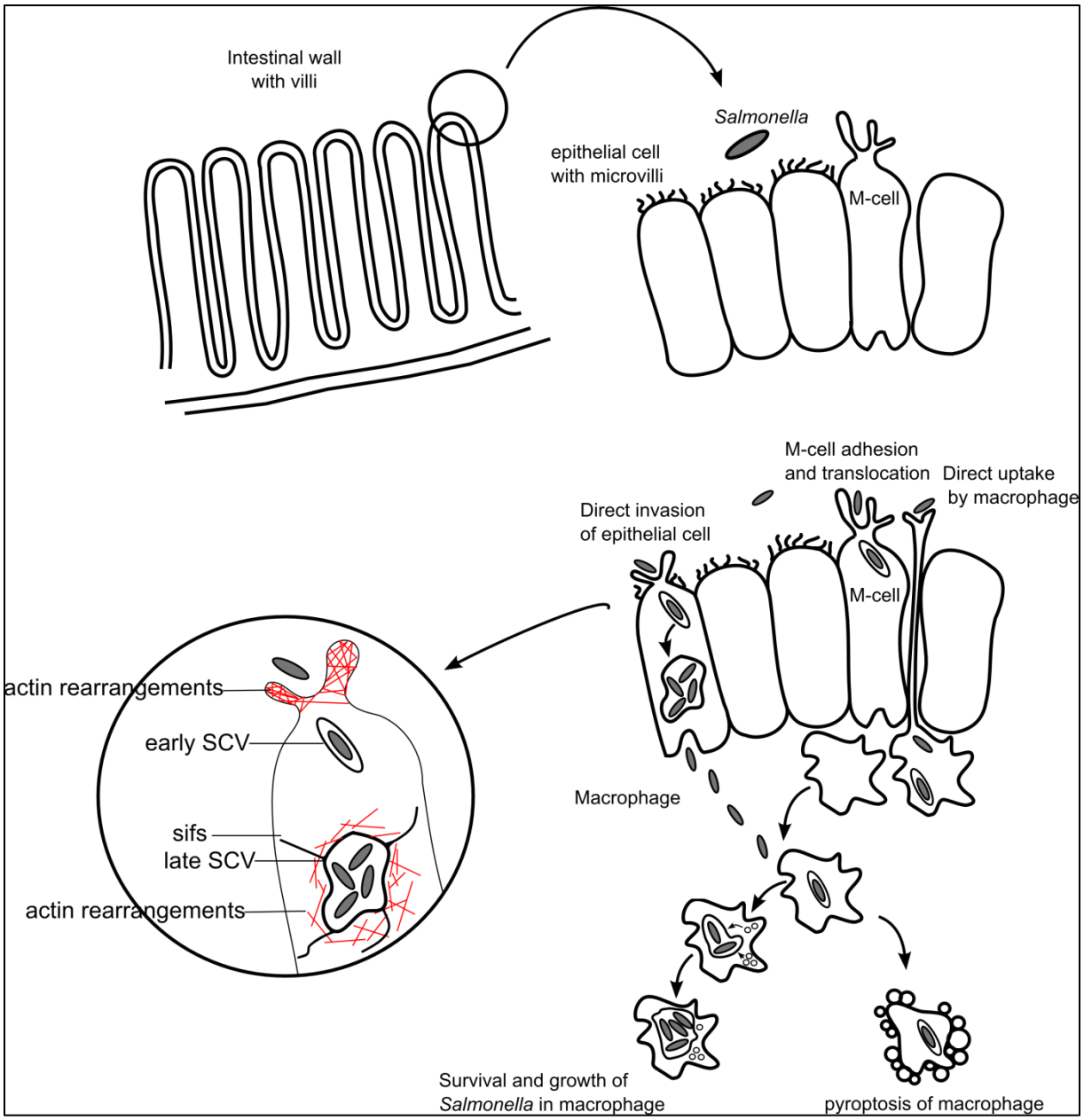


Figure 1.4: Virulence characteristics and pathogenesis of *Salmonella*. *Salmonella* can invade the host in at least 3 different ways. Through Phagocytosis by an microfold cell (M-cell), by direct uptake by a phagocyte or by active invasion of an epithelial cell. In the latter case, effectors of a first T3SS secreted by *Salmonella* induce the rearrangement of the actin skeleton and the formation of membrane ruffles followed by internalization of the pathogen. Effectors of a second T3SS induce the transformation of the phagosome into a *Salmonella containing vacuole* (SCV) in which the bacterium can survive and replicate. On the non-intestine side, the *Salmonella* bacteria may be taken up by macrophages which normally destroys harmful elements and dead cells. Here again, *Salmonella*, is able to enter and to form a vacuole where it is protected and can multiply by use of its second T3SS (Broz *et al.* 2012). In the macrophages, a.o. pyroptosis, a kind of programmed cell death, may induced which force the protected pathogens to be exposed again to extracellular immune defenses. Figure redrafted and adjusted from (Sansonetti 2004; Croxen and Finlay 2009; Torgersen *et al.* 2010; Broz *et al.* 2012, Ibarra, 2009 #1876).

1.4 Butterhead lettuce production

From the leafy vegetables, lettuce (*Lactuca sativa*, Asteraceae) is considered the most important. It is almost exclusively used as a fresh, raw vegetable in salads. The diverse varieties belong to seven groups of cultivars: butterhead lettuce, crisphead lettuce (iceberg lettuce), romaine lettuce (cos

lettuce), cutting lettuce, stalk (also called stem, Chinese or Asparagus) lettuce, latin lettuce and oilseed lettuce (Singh 2006; Křístková *et al.* 2008).

Butterhead lettuce (*Lactuca sativa* L. var. *capitata*) is the main crop type that is grown in Belgium. The crop can be distinguished from other varieties by its heavy crop weight (400-550 g), soft folded leaves and closed head formation. It is an important economic crop. The total Belgian production is around 100 million heads and the average consumption in Flanders is 2.2 kg per person a year. It is the second most exported fresh vegetable after tomatoes (lettuce: ± 60 million euro/year, tomato: ± 200 million euro/year). The main importers are, in order of importance, Germany, France and The Netherlands (Derden *et al.* 2005). Butterhead lettuce is mainly consumed in the summer and is mostly sold as an intact crop but also available as precut bagged lettuce.

The lettuce production chain starts with growing seedlings in nurseries until the 3-5 leaf stage is reached. Subsequently, they are planted in the field or in the greenhouse. In Belgium, butterhead lettuce is primarily cultivated in glass-covered greenhouses in Flanders (± 200 ha) (Pauwelyn 2011; Platteau *et al.* 2012). The crop is intensively cultivated in the ground in a continuous monoculture system or in crop rotation with tomato (Van Beneden *et al.* 2009). Seedlings are planted into the greenhouse 30 cm apart. Mainly inorganic fertilizer is used and the plants are commonly irrigated using overhead sprinkler irrigation with groundwater, pond water or collected rainwater but also tap water and water from a stream may be used (Pauwelyn 2011). Average cultivation conditions in winter are minimum 10°C at day and 5°C at night, with relative humidity not higher than 80-85%. During summer the temperature is on average kept on 10°C at night and 20°C during day, although sometimes 25°C is reached. The relative humidity is a bit lower between 70-75% on average (Van Laere 2009). The lettuce is mature and ready for harvest 5-12 weeks after planting, depending on the cultivation period (Pauwelyn 2011). The harvesting occurs by manually cutting the lettuce crops. The crops are subsequently transported on a conveyer belt to the packaging house, sorted (small heads and heads showing signs of decay are removed), the outer leaves are removed and the crops are rinsed in order to remove dirt, soil and to reduce microorganisms. Subsequently, the crops are transported and sold to the auction or directly to the processing industry (Holvoet 2014).

Alternatively, lettuce is grown in the open field, here, both inorganic or organic fertilizer such as manure can be used and irrigation is applied depending on the weather conditions. To a lesser extent, lettuce is grown hydroponically. The latter technique gains recently more attention due to the fact that production speed is higher (around 30%) and the lettuce crops are cleaner and can be sold with intact roots which has a positive influence on the shelf life (Heijboer 2010).

1.5 From field to plate: introduction routes of enteric pathogens to the vegetable production chain

Traditionally, the source-sink model describes the genesis of human infections with pathogenic *E. coli* and *Salmonella*. Following this model, organisms occupy two kinds of habitat, a source and a sink. The source, is defined as a high quality habitat that on average allows the bacterial population to increase. The sink, is a habitat that is only able to support a population due to immigration from the 'source' population. In some cases, the sink population can be maintained on its own, but only transiently, which means that the population is rather small with a high probability of extinction (Sokurenko *et al.* 2006; Pennington 2010). Following this model, domestic animals and wildlife are considered as the source of the pathogens and the environment and human as the sink.

It is usually accepted that enteric bacterial pathogens enter the agricultural environment via animal feces (Barak and Schroeder 2012). Feces, on their turn, may contaminate surface water, soil, compost, feed, crops and humans. The survival of the pathogen in these matrices is influenced by a variety of factors such as bacterial species and strain, the amount of bacteria (inoculum level), climatic conditions, competition with natural background microbiota and predation. Consequently, large variation in survival rates in these matrices is reported. The knowledge about the survival of the pathogens in these matrices are shortly discussed below. A scheme of the possible introduction routes of enteric pathogens to the vegetable production chain is shown in Figure 1.5.

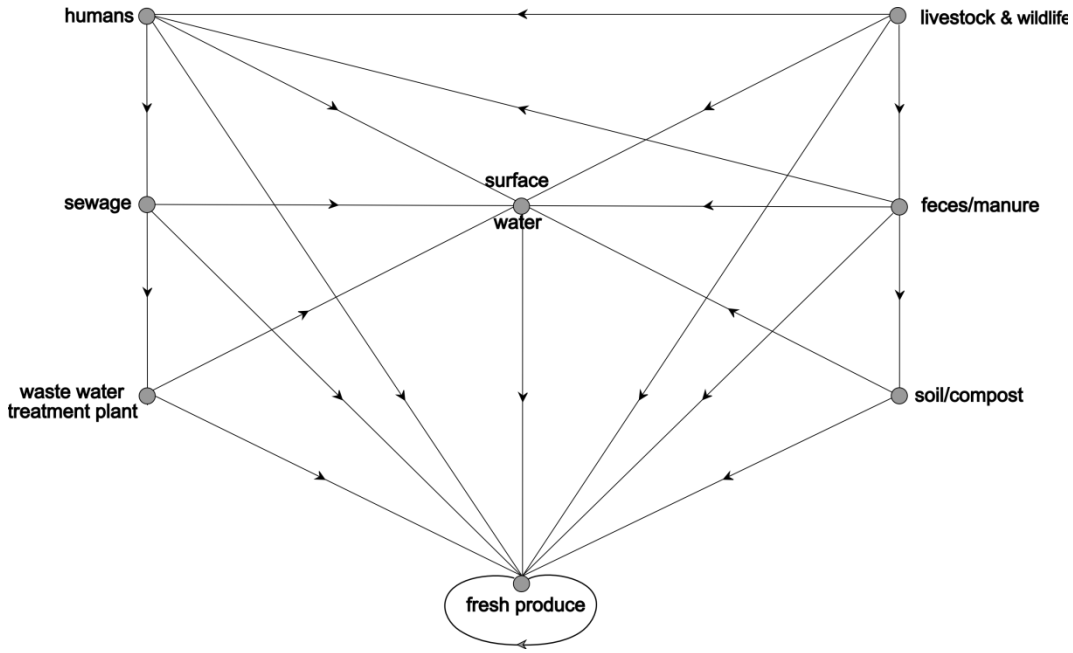


Figure 1.5: Scheme of possible introduction routes of enteric pathogens to the vegetable production chain.

1.5.1 Ecology and survival in feces, sewage, manure, soil and water

Contaminated feces may originate from livestock and wildlife but also from humans. They may contain between 10^2-10^6 CFU/g *E. coli* and 10^2-10^7 CFU/g *Salmonella* (Himathongkham *et al.* 1999; Tenaillon *et al.* 2010). Feces may contaminate the produce directly. During a preharvest contamination this can be caused through shedding of wild animals. Wild swine and cow feces were e.g. probably involved in the *E. coli* O157:H7 outbreak with spinach, in the USA in 2006 as discussed previously (1.2.1). But also smaller animals such as snails and mice, obtain the possibility to spread pathogens via defecation (Semenov *et al.* 2010). Also insects may act as a vector during pre- and postharvest processes (Erickson *et al.* 2010a). In a postharvest situation, produce can be contaminated through improper handling by an infected food handler with poor hygiene. Survival in feces was mainly tested in lab scale experiments and highly dependent on the initial pathogens level and tested conditions. Survival up to almost 2 years was e.g. recorded for *E. coli* O157:H7 (Kudva *et al.* 1998). As a result, the pathogens may be present in fecally derived material such as manure (animal origin), sewage (human origin) and their respective sludge (settled out solid matter) and slurry (fluid mixture). Generally, the survival of both pathogens in fecally derived material decreases over storage time and is enhanced with aeration and higher temperatures (Whipps *et al.* 2008). Survival rates of more than 4 months were e.g. recorded for both *E. coli* O157:H7 and *Salmonella* in manure (Franz *et al.* 2005). These fecally derived materials can be applied on the growing fields as fertilizers. But normally, direct contact of manure with vegetables should not occur if either a treatment step (e.g. composting, aerobic and anaerobic digestion, drying) or a significant time interval between application and crop production is considered. These practices are often regulated by governmental agencies (Warriner *et al.* 2009). In Belgium, the requirements regarding manure application are laid down in the IKKB standard (Vegaplan 2013) which is approved and recognized by the Belgian food safety agency to encompass all minimum legislative requirements. Manure that is intended to be used at the production unit, needs to be of natural origin originating from the own production unit, from a third farmer or allowed fertilizer. For fertilizers derived from waste, special (regional) guidelines exists with more stringent guidelines for purification sludge. Purification sludge cannot be applied on fields with growing potatoes and vegetables or fruits. For orchards there is an exception and purification sludge can be applied between harvest and flowering. Furthermore, purification sludge must be applied at least 10 months before harvest on grounds on which fruits and vegetables which comes in contact with the ground will be grown and which are usually consumed raw. The use of sewage sludge on agricultural ground is not allowed in Flanders region. Certification to IKKB standard is a prerequisite to be allowed to deliver lettuce crops to the auctions and further sales to major retail shops or fresh-cut lettuce processing companies (Holvoet 2014). Manure

intended for export, needs to fulfill a.o. European requirements ((EG) 1774/2002) which lay down requirements regarding *Salmonella* (absence in 25g), and *Enterobacteriaceae* (less than 3 log CFU per gram of treated product). The manure must be subjected to a treatment of 1 h at 70°C or an equivalent treatment.

When fecally derived material is not treated or stored properly, pathogens may contaminate the soil and subsequently the plants through splashes or via the roots. The survival of *E. coli* O157:H7 and *Salmonella* in the soil is, similarly to the survival in feces and fecally derived material, mainly tested under lab conditions. Here again, highly variable results are recorded. Soil type and physicochemical characteristics (pH, exchange capacity, water holding capacity, organic content, nutrient levels, UV) could be found as influencing factors. Reduced pathogen survival was e.g. observed in sandy soils in comparison with clay soils. Higher temperatures result in increased die-off. Also the method of application may influence the pathogen's survival. For *Salmonella*, reduced survival was observed after surface application of manure in comparison with injection in the soil which is often applied due to air pollution problems. For *E. coli* O157:H7, this application method effect could not be observed (Semenov *et al.* 2009). Furthermore, competition with the background microbiota may reduce the survival of the pathogen, whereas protozoa and helminthes may increase the survival (Brandl *et al.* 2005a; Gourabathini *et al.* 2008; Lacharme‐Lora *et al.* 2009). Interestingly, the presence of plants growing in the soil can also enhance survival (Gagliardi and Karns 2002; Semenov *et al.* 2009) (see also 1.6.1). *Salmonella* sp. has been reported to survive from a few days up to 332 days in manure-amended soils, whereas the survival of *E. coli* O157:H7 has been reported from a few days up to 193 days (Jacobsen and Bech 2011). Confirmed outbreaks related to soil as a vector and reservoir of enteric pathogens are, however, scarce.

But water is considered as the most likely key route of dispersal from the feces to the environment and from environment to plants (Barak and Schroeder 2012). Contaminated animal fecal material can be deposited directly into surface water or transported into surface water by runoff or through leaching through the soil following rain or flood events (Gagliardi and Karns 2000; Barak and Schroeder 2012). Contamination trough human feces can be caused by discharge of water from a malfunctioning waste water treatment plant. Contaminated irrigation water in his turn, may contaminate the soil. Before harvest, water can come in direct contact with crops in two ways: by irrigation, and by the often overlooked, pesticide or fertilizer application. After harvest, produce can get cross-contaminated during washing.

Experiments on survival of enteric pathogens in natural waters usually show a decrease in microbial concentrations with time, although in some cases an initial increase of pathogen level can be observed, especially under warm conditions. Temperature seems to be the most important factor

influencing pathogens' survival but also nutrient, pH, radiation and presence of other micro- and macrobiota. An important conclusion made by van Elsas *et al.* is that with the current state of knowledge accurate predictions of the survival patterns for specific pathogens in specific irrigation water or microbial reservoirs, cannot yet be made, even though factors of pathogen survival and patterns of pathogen microbial population changes in time are generally known (van Elsas *et al.* 2010; Pachepsky *et al.* 2011). Although, irrigation water is generally considered as a potential source of contamination, again there are only relatively few confirmed cases from outbreaks resulting from contaminated water. Three outbreaks with *Salmonella* and tomatoes in the USA could be linked to contaminated irrigation water. One outbreak occurred in 2002 and two in 2005, the outbreak strains were isolated from the ponds that were used to irrigate the fields. The serovar was each time *Salmonella* Newport which suggests that contamination can be persistent. In 2005, Söderstrom *et al.* linked an *E. coli* O157:H7 outbreak with lettuce in Sweden to irrigation water as well. The lettuce was irrigated from a small stream and identical Vero toxin producing *E. coli* O157:H7 strains were isolated from the patients and the cattle at a farm upstream from the irrigation point (Söderström *et al.* 2008). Accidental mixing of well water, intended for irrigation, with water from a dairy manure lagoon was shown to be the cause of another *E. coli* O157:H7 outbreak associated with shredded lettuce in 2008 in the USA. Furthermore, *Salmonella* Saintpaul was found in irrigation water and in Serrano peppers implicated in an outbreak (Pachepsky *et al.* 2011). Irrigation water was suspected to be the source for a lot of other outbreaks, but, for these outbreaks proof by reisolation of the pathogen could not be obtained (e.g. (Ackers *et al.* 1998; Hilborn *et al.* 1999)). Although the potential for produce contamination from irrigation water has been established, it is still difficult to quantify the extent of the problem.

In Belgium, irrigation water for lettuce production may be pond water, groundwater, tap water, water originating from a stream or collected rain water. Furthermore, some processing water may be reused as irrigation water: wash water from fruits and vegetables (except for wash water from carrots and turnips), water from blanching/sterilization treatments or quick freezing and water used to clean sterilization/blanching installation. No requirements are set, but the federal food safety agency FAVV recommends to use water only as irrigation water if the water has a maximum load below 3 log CFU *E. coli*/100 ml (Federaal Agenschap voor Veiligheid van de Voedselketen (FAVV) 2009). For the rinsing water, requirements are defined in the IKKB standard. For vegetables intended for direct consumption the last rinse should be performed with water with drinking water quality, the previous rinses can be done with so called 'schoon' water (maximum 4 log CFU *E. coli*/100 ml., Regulation (EC) No 852/2004). The last rinse of vegetables intended to be processed before

consumption should be rinsed with 'schoon' water. For sprouts intended for consumption, water with drinking quality should be used throughout the whole process.

1.5.2 Bacterial colonization of plants

For both pre- and postharvest contamination events, colonization of the plant is a prerequisite. Bacterial colonization of plants in general involves different steps: attachment, adaptation, proliferation and possibly internalization. Depending on the introduction route, bacteria can be found on roots (rhizosphere) and/or leaves (phyllosphere). This thesis focusses on contamination of the leaves, as this is the part of lettuce that is consumed. Therefore, the interaction between phyllosphere bacteria, also called epiphytes, and the plant will be discussed in further detail. When bacteria (commensals, symbionts, plant pathogens or human pathogens) arrive by air, water, or another vector on the plant, this results in a random distribution of the bacteria across the leaf surface. In order to colonize the leaves, the bacteria need to attach to the plant tissue. This attachment process consists of two phases. Initially, reversible attachment occurs and the bacterial cells can be removed from the plant by rinsing or a turbulent flow. This initial attachment is mediated by basic forces such as the Van der Waals force, electrostatic force and hydrophobic/hydrophilic force (Goulter *et al.* 2009). Then, irreversible attachment may take place, enabled by the use of cellular surface structures (such as flagella or fimbriae) or excretion of extracellular polysaccharides. Subsequently, the epiphytic bacteria need to adapt in order to survive the large and rapid fluctuations in environmental conditions on the leaf such as UV radiation and water availability. They can do this by modifying their local environment by enhancing nutrient release from the plant or again by producing extracellular polysaccharides. Once established, they will be able to divide and form microcolonies which may eventually develop into large aggregates. When high density is reached, some of these bacteria (especially plant pathogens but enteric pathogens as well, see also 1.6.1) may establish internal populations. They can egress again by water-soaking or lesion formation (Beattie and Lindow 1999; Lindow and Leveau 2002; Lindow and Brandl 2003; Lindow 2006). In order to find out the importance of factors that mitigate or enhance the survival, attachment, internalization of enteric pathogens on fresh produce, a lot of research was performed. The present knowledge about the interaction of *E. coli* O157:H7 and *Salmonella* with plants, more specifically lettuce, is discussed in the next section.

1.6 Schematic overview of the existing research on the interaction between enteric pathogens and lettuce

1.6.1 Schematic overview

During the last 20 years, a lot of effort was done to understand the contamination of fresh produce with enteric bacterial pathogens. To give an overview of the state-of-the art of the scientific knowledge and to be able to discover the research gaps regarding this topic, a literature review, which focusses specifically on the contamination of lettuce with *E. coli* O157:H7 and *Salmonella,* was performed. Therefore, all scientific articles published between 1993 and march 2013 with keywords '*E. coli* 0157:H7 and lettuce', '*Escherichia coli* O157:H7 and lettuce' or '*Salmonella* and lettuce' were exported from the ISI Web of Knowledge Database (http://apps.webofknowledge.com). Reviews, studies investigating methodology, monitoring studies, specific outbreak related studies and studies only investigating the behavior of non-pathogenic *E. coli* were not taken into account. This resulted in a total of 180 research articles. A total of 28 different research questions were identified (Figure 1.7) and for each scientific study the different research questions were inventoried. These questions could be divided into questions concerning the influence of bacteriological, plant, environmental or processing factors on the survival, attachment and internalization of the pathogen on the crop. Furthermore, research could be split up in pre- or postharvest research or a combination of both. The data were inventoried in a Microsoft Access database, analyzed in Microsoft Excel and visualized in Microsoft Excel (Figure 1.6, Figure 1.7) and Gephi (Figure 1.8). The results are discussed with special attention towards preharvest research. As a lot of outstanding research was also performed on fresh produce other than lettuce, the most important conclusions of these studies are also mentioned.

Investigated pathogens

More studies were performed with *E. coli* O157:H7 (76.1%) in comparison with *Salmonella* (39.4%). Apart from *Salmonella* and *E. coli* O157:H7, the behavior of *Listeria monocytogenes* (20.5%) and non-pathogenic *E. coli* (11.1%) were most frequently examined. Up to four different species could be investigated simultaneously.

Lettuce

The lettuce cultivars that were investigated were among others romaine lettuce, iceberg lettuce, green leaf lettuce, red leaf lettuce, rocket lettuce, mixed lettuce and butterhead lettuce (Figure 1.6). Two lettuce varieties were more intensively investigated: romaine lettuce and iceberg lettuce. In preharvest research, romaine lettuce and iceberg were equally investigated (in 33.3% vs. 29.3% of

the studies) whereas in postharvest studies the main focus lied on iceberg lettuce (22.9 vs. 48.8%). Butterhead lettuce was less frequently used (5.3% in preharvest research, 4.1% in postharvest research). The difference between different lettuce varieties was investigated in 12.2% of the articles, in 22.2% of the studies also other fresh produce such as spinach, tomatoes, basil, apple, etc. were investigated together with lettuce.

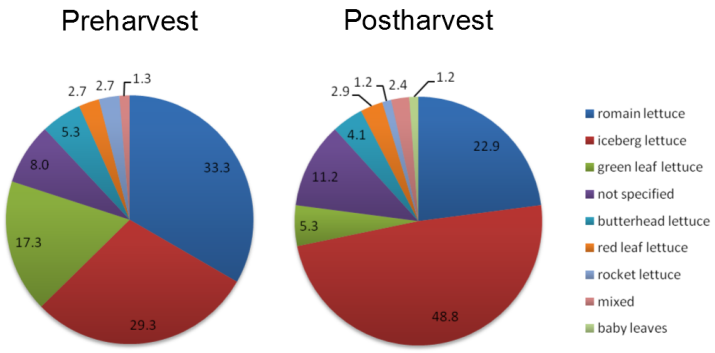


Figure 1.6: Overview of the investigated lettuce varieties in preharvest (left) and postharvest (right) studies. The numbers in the segments of the circles represent the percentage of the studies (77 in total for preharvest, 130 in total for postharvest) investigating the particular lettuce crop type.

Pre- or postharvest contamination event

The analysis showed that almost two third of the selected articles (57.2%) was focused on postharvest contamination processes, 27.8% on preharvest contamination and 15.0% could be assigned to both pre- and postharvest research. The latter were mainly research articles in which more fundamental questions (such as investigation of the attachment mechanism of the pathogens) were treated and which were applicable for both pre- as postharvest contamination events. Only a few studies have followed the behavior of the pathogen through the whole production process from preharvest until retail and consumption (Oliveira *et al.* 2011).

Survival and growth studies

For preharvest research, the survival and growth studies were the most important (40.3%). In these studies, the pathogen level on the plant was monitored during growth of the plants. The main bacteriological factor that was investigated was the difference in survival between different pathogen species. The influence of different plant factors was the second most important investigated factor. Studies were performed on all growth stages of the plant and all kinds of plant parts: seeds, seedlings, roots, leaves, fruits. Only a few studies focused on seed as contamination source. It was shown that once contaminated lettuce seeds germinated, the pathogens could multiply on the seedlings (Wachtel *et al.* 2002). Moreover, in one study (not included in the analysis) on spinach seeds inoculated with high amounts of non-pathogenic *E. coli*, it was shown that the

bacterium could be recovered from the roots and leaves of the plants up to 7-weeks after sowing (Warriner *et al.* 2003a). Much more emphasis laid on rhizosphere contamination through contaminated soil or manure. It was shown that the pathogens could persist for long time in the soil and, interestingly, survived significantly longer when also plants were growing in the soil (Islam *et al.* 2004a). This indicates that vegetation appears to provide a protective environment for the bacteria. Furthermore, *E. coli* O157:H7 seems to be attracted by the root exudate of the plants (Klerks *et al.* 2007). Moreover, the pathogens are also able to move on the plant. When high inocula were used, plants planted in contaminated soil could harbor pathogens on the aerial parts of the plants up to harvest (Islam *et al.* 2004a; Islam *et al.* 2004b).

Survival in the phyllosphere was less intensively studied and seemed to be significantly lower compared to the rhizosphere (Warriner and Namvar 2010). The literature analysis showed that when high inocula were used to inoculate plant leaves (log 6-8 CFU/g lettuce), a decline of the pathogen was often seen, although the pathogen could be detected throughout the cultivation of the plants up till harvest (Solomon *et al.* 2002). When lower, more realistic, inoculum densities were used, the results were more variable: growth of the pathogen could be observed on the leaves in favorable conditions (Solomon *et al.* 2003; Brandl and Amundson 2008) but also a decline of the pathogen could be observed. Many factors may influence pathogen survival and due to the extensive variance in experimental design, it is still not completely clear which combination of factors promote growth/no growth of the pathogens on plants. The pathogen survival seems to be different between different vegetables but also between lettuce cultivars. Also within one lettuce cultivar variance could be observed (Quilliam *et al.* 2012) and even within one lettuce crop type, the survival could be significantly different between different crop stages, leaves and between the ab- and adaxial side of the leaf (a.o. (Brandl and Amundson 2008; Zhang *et al.* 2009)).

Environmental conditions have an important influence on the survival of the pathogens on fresh produce as well. For preharvest research the influence agricultural practices such as light conditions, relative humidity, amount of irrigation and irrigation system, (drip, sprinkler), application of nitrogen and cultivation system (greenhouse, open field, hydroponics) was investigated. Research, not specifically related to lettuce, revealed that higher temperatures such as 30°C might increase the competitiveness of the pathogen and thus their survival. Also high relative humidity (Brandl and Mandrell 2002) and wounding of the tissue, e.g. made during harvest, could increase the persistence of *E. coli* O157:H7 and *Salmonella* on the plant (Barker-Reid *et al.* 2009; Harapas *et al.* 2010). Similar results were found with injuries caused by phytopathogens (Brandl 2008). The majority of the preharvest survival studies were performed in the controlled environment of a scientific plant growth chamber or in the laboratory (67.3%).

Also postharvest research is dominated by survival studies, but the perspective lays dominantly on the investigation of the efficacy of sanitation treatment (79.6%) and the effect of storage temperature (22.3%). Especially the efficacy of different sanitizers were tested on different lettuce varieties or other fresh produce. This focus on decontamination may be explained by the fact that most research was done in the USA, where sanitation is routinely used for postharvest cooling water, in postharvest treatments and during rehydration at shipping destinations (Suslow 2005). Here again, pathogen survival after decontamination seems to be leaf side dependent (Koseki *et al.* 2003). Furthermore, it is generally accepted that decontamination may reduce the pathogen level but not eliminate the pathogen completely.

Attachment studies

In the attachment studies, factors which influence the ability of the pathogen to attach to a plant were investigated. Most attachment studies were more fundamental and could be investigated regarding preharvest and postharvest. It was shown that there were differences in attachment between the different pathogen species, but also between different strains of the same species. *E. coli* O157:H7 seemed to attach preferentially to cut edges whereas *Salmonella* seemed to attach equally to surface and cut edges (Takeuchi *et al.* 2000). Also vegetable type and leaf part seemed to play an important role (Barak *et al.* 2008; Patel and Sharma 2010; Kroupitski *et al.* 2011). Studies with mutant bacteria revealed that cell surface components such as cellulose, flagella, pili with Type III secretion systems may play a role, although strain variability was observed as well (Barak *et al.* 2005; Jain and Chen 2007; Shaw *et al.* 2008; Goulter *et al.* 2010; Patel *et al.* 2011; Shaw *et al.* 2011). Studies with *Salmonella* and *E. coli* O157:H7 showed that in contrast to most phytopathogens, cells do probably not associate with biofilm structures but tend to aggregate between the grooves of epidermis cells and to leaf structures such as trichomes (Warriner *et al.* 2003a). Pathogens could also be found in the protective niche of the stomata. These natural openings in the leaves, which enable gas exchange, are thought to be one of the introduction routes for the pathogens to be established in the interior of the leaf (internalization).

Internalization

The cells that penetrate the tissue cannot effectively be killed by sanitation, therefore, a lot of effort was done to search for the factors which may influence internalization (Takeuchi and Frank 2001). Apart from through stomata, the pathogens seems to be able to enter plant tissue through lateral junctions of roots and damaged tissue (wounds, cut surfaces) both before and after harvest.
Very recently, it was also shown that *E. coli* O157:H7 was able to internalize inside the cell wall of epidermal and cortical cells of spinach and *Nicotiana benthamiana* roots (Wright *et al.* 2013).

Internalization was mainly observed when the plant tissue was inoculated with a high inoculum (≥6 log CFU/g soil or 6 log CFU/ml water). Studies which use lower inocula could only detect internalization sporadically. It also seems that internalization via the roots or seeds is not likely to occur when the plants are cultivated in soil systems, this in contrast with hydroponics (Erickson 2012). Internalization through the rhizosphere is extensively reviewed in Hirneisen *et al.* (Hirneisen *et al.* 2012).

One of the processes underlying internalization into the leaves was revealed for *Salmonella* by Kroupitski *et al.* (Kroupitski *et al.* 2009). They showed that *Salmonella* needs to be motile to be able to internalize into iceberg lettuce and that attraction to nutrients (mainly fructose) produced by photosynthetically active cells was the driving force. But again, lettuce crop type seems to be important, these results could not be observed for romaine lettuce (Golberg *et al.* 2011).

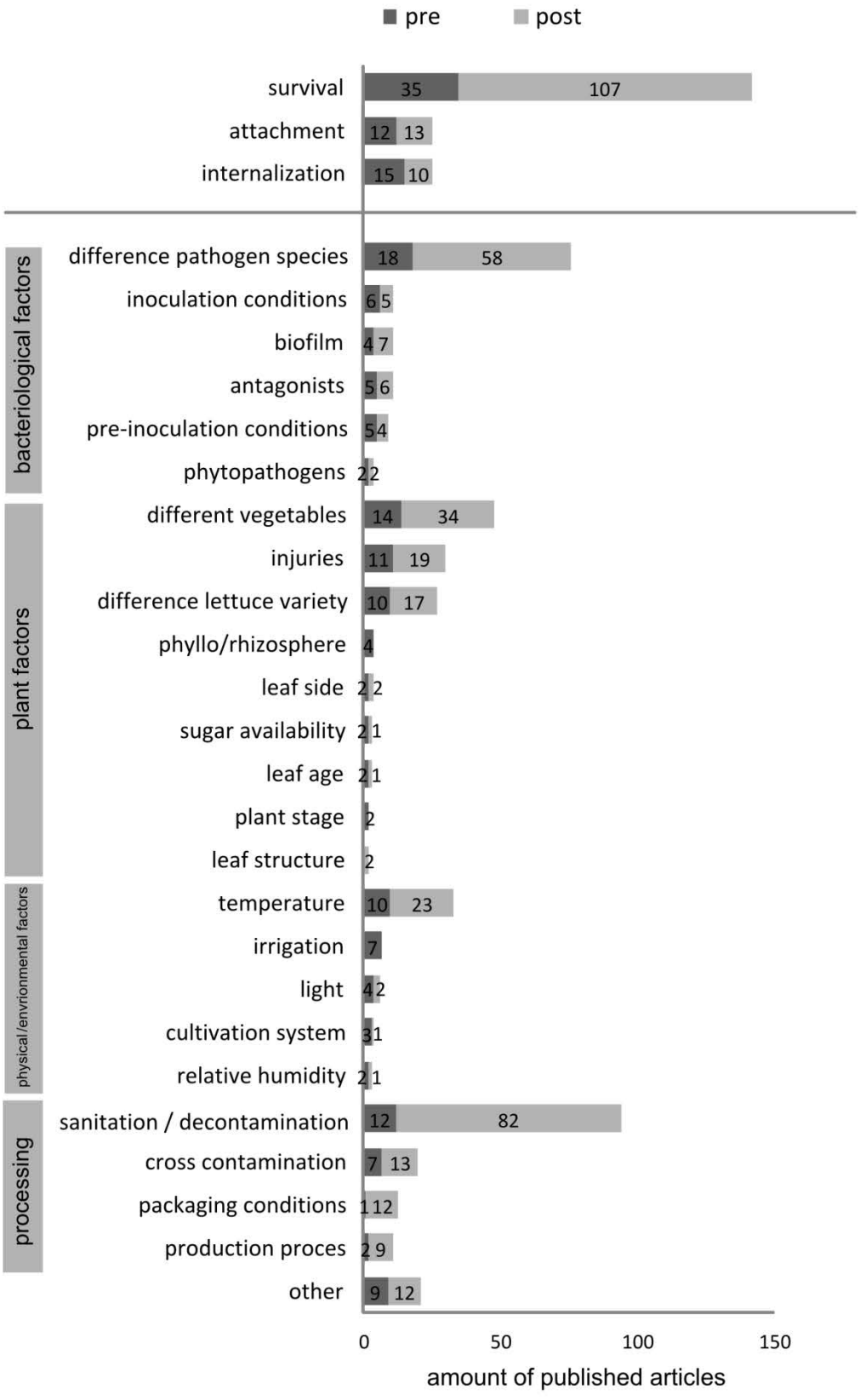


Figure 1.7: Overview of the investigated factors in pre- and postharvest studies (n=180). Each bar shows how many times each factor was investigated in preharvest and postharvest research respectively.

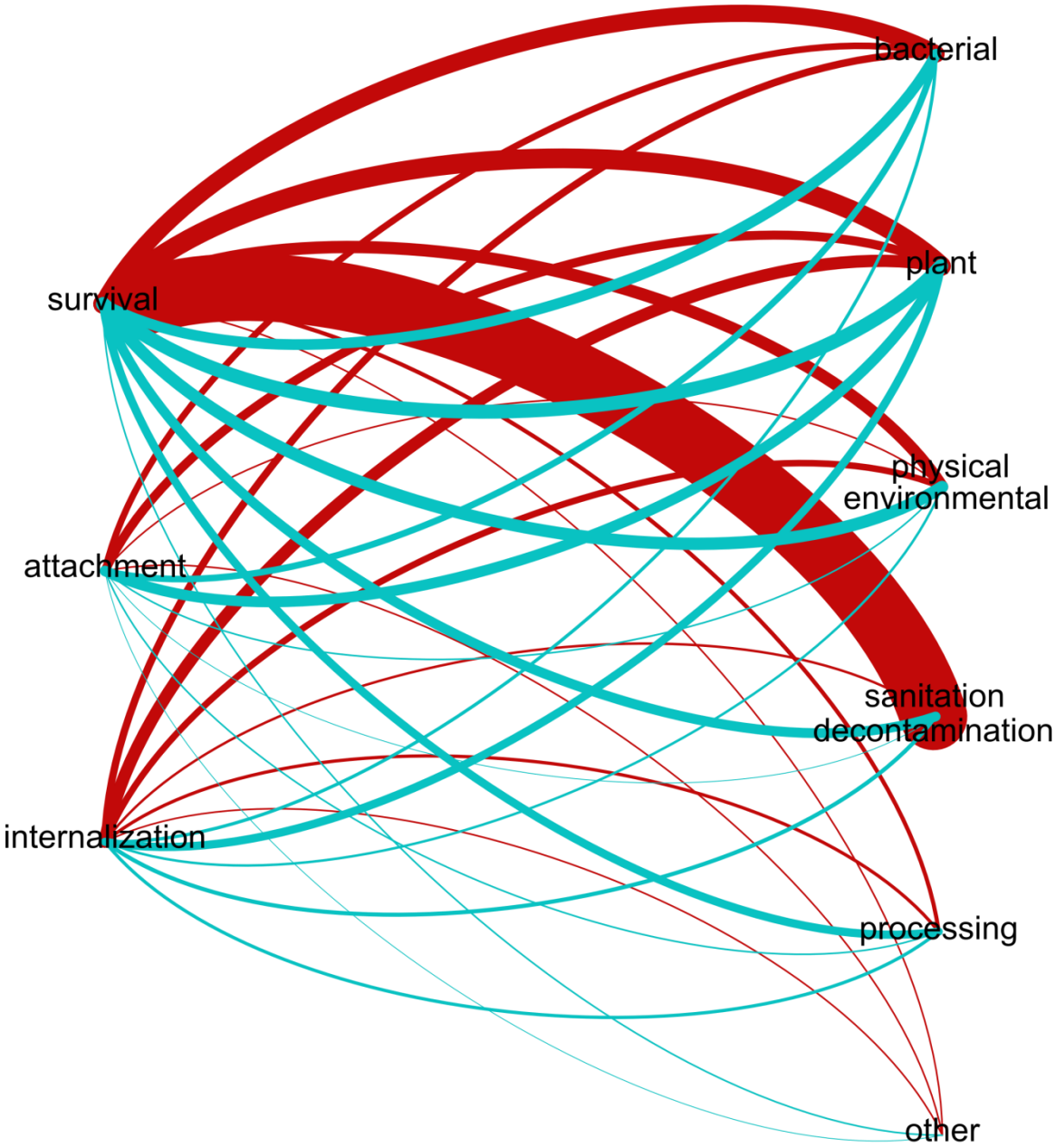


Figure 1.8: Network graph which shows how often two factors were investigated together in one research article. The factors are grouped by their factor classes. Each investigated factor class is shown as a node. The lines between the nodes, called edges, link two factor classes which were investigated simultaneously in one research article. The thickness of the edges represent the number of research papers investigating the link between the two factor classes. On the left side, the factors which describe the behavior of the pathogen on the plant (survival, attachment and internalization), on the right side the bacteriological, plant, physical, processing and other factors are shown. Sanitation/ decontamination (which is classified as a processing factor in Figure 1.7) is shown as a separate node in order to show the importance of this factor in the investigated research articles. Green: preharvest research. Red: postharvest research.

1.6.2 Research gaps and research goals

Lack of information about the European vegetable production conditions.

The majority of the previous research was performed in the USA. Consequently, these studies are adjusted to the vegetable production conditions of the USA (Salina Valleys, California) which are quite different from the Belgian/European condition regarding lettuce type, production system, climatological conditions but also legislation. As this kind of research is relatively new, the majority of the preharvest survival studies was performed in the controlled environment of a scientific plant growth chamber or in the laboratory (67.3%), with high temperatures and relative humidity conditions. Researchers only started recently to mimic the contamination event more closely, therefore, only 8.2% of the preharvest studies were performed in the greenhouse, 24% in the open field. The climatological conditions in the greenhouse are different from the field. The most important difference is that the plants are protected from UV light, which is known to have a bactericidal effect. The main focus of the PhD study will, therefore, be on greenhouse production of butterhead lettuce, a lettuce type which is a typically greenhouse grown northern European type. The emphasis was put on preharvest survival of the pathogen, as there is no possibility to kill the pathogen later in the production chain by decontamination. Especially the introduction of the pathogen by contaminated seed (Chapter 2) and irrigation water (Chapter 3) will be studied as contamination through manure is considered to be less important as inorganic fertilizers are mainly used in Belgian greenhouse production.

Lack of information about the influence of stress that the pathogen likely may encounter in the field before contamination of the produce.

It is remarkable that almost all studies were performed with unstressed pathogens. The pathogens were generally grown in nutrient rich broths such as Luria Bertoni broth (LB) or tryptic soy broth (TSB) and cultured overnight at 37°C with a few exceptions where the pathogens are grown in a minimal medium at lower temperatures (18°C-28°C) e.g. (Cooley *et al.* 2006; Brandl and Amundson 2008) (see also 7.6). Consequently, the pathogen is actively growing at the moment that it is artificially inoculated into the plant environment (leaves, seed, roots). This is in contrast with the general idea that the pathogen is in survival mode before it comes in contact with the plant environment (see 1.5). Therefore, efforts will be done to better simulate the stress that the pathogen may experience before it comes in contact with the plant environment. The influence on their survival (Chapter 4) and attachment (Chapter 5) will be investigated.

Lack of information about the underlying genetic mechanism for the enteric bacterial survival in a plant environment.

Finally, the basic mechanisms that the pathogens use to survive in a plant environment are still not well understood. Although a restricted number of studies have investigated the molecular mechanisms that the pathogens use to survive, attach or internalize on a plant by e.g. gene expression studies, mainly postharvest experiments were conducted. The preharvest situation will, therefore, be investigated in Chapter 6.

Chapter 2:

Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butterhead lettuce seeds, and their subsequent survival and growth on the seedlings

Redrafted from:

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., & Heyndrickx, M. (2013). Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butterhead lettuce seeds, and their subsequent survival and growth on the seedlings. International Journal of Food Microbiology, 161, 214-219.

Germination of 2-year old contaminated lettuce seeds at day 3, 5, 7 and 11

Abstract

The long-term survival of enteric pathogens on butterhead lettuce seeds, and their subsequent survival and growth on seedlings was investigated. Lettuce seeds were inoculated at a high level with two *Salmonella enterica* and two *Escherichia coli* O157:H7 strains each (± 8 log CFU/g seed) and the survival of the pathogens was monitored over two years using standard plating techniques on selective medium. The *Salmonella* strains (serovars Typhimurium and Thompson) survived significantly better on the seeds than the *E. coli* O157:H7 strains (MB3885 and NCTC12900). When individual seeds were tested two years after inoculation, *Salmonella* was recovered from each individual seed, whereas *E. coli* O157:H7 only from 4% to 14% of the seeds, depending on the recovery method. When contaminated stored seeds were germinated and the seedlings examined for presence of the pathogens, it was clear that both pathogens were able to proliferate on the seedlings. Pathogen counts up to 5.92 log CFU and 4.41 log CFU per positive seedling were observed for *Salmonella* and *E. coli* O157:H7, respectively. Our study not only confirms the long-term survival of enteric pathogens on seeds but also shows that the pathogens maintain their ability to resuscitate and proliferate on the seedlings. Seeds or seedlings should be considered as contamination sources for the cultivation of leafy vegetables such as butterhead lettuce grown in greenhouses.

2.1 Introduction

The ability of enteric pathogens to contaminate nonmeat products is an accepted fact (Holden *et al.* 2009). This is shown by the numerous outbreaks with enterohaemorrhagic *Escherichia coli* (EHEC) and *Salmonella enterica* that have been linked to consumption of contaminated fresh produce such as cantaloupe, herbs, lettuce, tomatoes, spinach and sprouts. In the USA, leafy vegetables such as spinach and lettuce are the most frequently implicated fresh produce (Rangel *et al.* 2005) and in Europe (EU), at least three *E. coli* O157 (Welinder-Olsson *et al.* 2004; Friesema *et al.* 2008; Söderström *et al.* 2008) and three *Salmonella* outbreaks (Ward *et al.* 2002; Horby *et al.* 2003; Nygård *et al.* 2008) were related to lettuce in the past 12 years. Although contaminated irrigation water and manure (specifically for open air cultivation) are important sources for the preharvest contamination of lettuce (CFERT 2007; Söderström *et al.* 2008), contaminated seeds or their seedlings may also be a potential transmission route, which may be specifically important for greenhouse crops, but only a few studies have focused on it. These studies have investigated (i) the effectiveness of disinfection (Warriner *et al.* 2005; Trinetta *et al.* 2011) and (ii) the potential of the pathogens on contaminated seeds to persist on the mature plants and/or to internalize the different plant parts (Cooley *et al.* 2003; Warriner *et al.* 2003a; Jablasone *et al.* 2005; Warriner *et al.* 2005; Habteselassie *et al.* 2010). These studies have shown that both *E. coli* O157 and *Salmonella* could be detected on the

harvestable plants and in some cases they were also able to internalize into the root tissue.
Contaminated seeds have also been identified as the main source for outbreaks related to the consumption of sprouts and sprouted seeds such as the EHEC-outbreak in Japan in 1996 (Michino *et al.* 1999; Watanabe *et al.* 1999) and the outbreak in Germany and France in 2011 where *E. coli* O104:H4 was attributed to contaminated fenugreek seeds (EFSA and ECDC 2011; EFSA 2012).

The effect of sanitation techniques (Bari *et al.* 2008; Chang *et al.* 2010; Neetoo and Chen 2010; Zhao *et al.* 2010; Bang *et al.* 2011a; Bang *et al.* 2011b; Neetoo and Chen 2011; Nei *et al.* 2011; Zhang *et al.* 2011) or the growth of enteric pathogens during the sprouting process (Howard and Hutcheson 2003; Jablasone *et al.* 2005; Singh *et al.* 2007; Deering *et al.* 2011; Fransisca *et al.* 2011) or a combination of these approaches (Jaquette *et al.* 1996; Warriner *et al.* 2003b; Liao 2008; Fransisca *et al.* 2011) were mainly studied on alfalfa, white radish and mung been seeds and sprouts. Little is known, however, on the long-term survival of enteric pathogens on seeds. To our knowledge, only few studies reported on the survival of *E. coli* O157:H7 and/or *Salmonella* on alfalfa seeds (Taormina and Beuchat 1999; Wu *et al.* 2001; Beuchat and Scouten 2002), and few reports on the survival of *Salmonella* on nuts such as almond and pecans (Beuchat and Heaton 1975; Uesugi *et al.* 2006; Beuchat and Mann 2010). These studies have investigated different factors for the pathogen's survival: the influence of temperature (Beuchat and Heaton 1975; Uesugi *et al.* 2006), chemical treatment (Taormina and Beuchat 1999), inoculum level (Uesugi *et al.* 2006), the combined effects of water activity (Aw), chemical treatment and temperature (Beuchat and Scouten 2002), and detection methods (Wu *et al.* 2001). The studies have shown that *E. coli* O157:H7 and *Salmonella* can survive for a prolonged period (up to 54 weeks) on seeds and nuts.
Both cultivar seeds and seeds intended for the production of sprouts may be stored for several years before use, e.g the fenugreek seeds from the German and French outbreak in 2011 were imported from Egypt in 2009 and 2010 (EFSA and ECDC 2011; EFSA 2012). Therefore, it is important to know whether enteric pathogens could also colonize sprouts or seedlings after germination of long-term stored seed. To our knowledge only one study has shown that *Salmonella*, present on naturally contaminated alfalfa seeds, is able to resuscitate and grow on the sprouts after eight years of storage of the seeds (Fu *et al.* 2008). However, no information is available on the long-term survival of enteric pathogens on butterhead lettuce seeds and it is not known whether *E. coli* O157 is able to colonize sprouts or seedlings after germination of long-term stored seed. The aim of the present study is to examine the long-term survival on butterhead lettuce seeds of different strains of enteric pathogens (*E. coli* O157:H7 and *Salmonella*), and their subsequent survival and growth on their seedlings. For this purpose, three methods for the detection of the pathogens from seeds were investigated.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

Two *S.* enterica (serovar Thompson and Typhimurium) and two enteropathogenic *E. coli* O157:H7 strains were used. *Salmonella* Thompson RM1987N is a spontaneous nalidixic acid-resistant mutant of *Salmonella* Thompson strain RM1987, kindly donated by Dr. Maria Brandl (USDA-ARS, Albany, California, USA). Strain RM1987 is a previously described clinical isolate from a patient in a cilantro-linked outbreak in California (Brandl *et al.* 2005b). *Salmonella* Typhimurium PT 120/ad MB4880 (MB collection of the molecular bacteriology lab of ILVO-Technology & Food Science Unit, Melle, Belgium) was isolated from overshoes at a pig farm in Belgium. *E. coli* O157:H7 MB3885 was isolated from beef carpaccio and kindly donated by the Scientific Institute for Public Health (Brussels, Belgium) and *E. coli* O157:H7 NCTC12900 by Dr. Martin Woodward (Department of Bacteriology, VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom). Both *E. coli* O157:H7 isolates lack Shiga toxin genes (*stx1* and *stx2*) and were used as non-toxigenic surrogate strains for the Shiga toxin producing (STEC) serotype O157:H7 for biosafety reasons. For *E. coli* O157:H7 MB3885, the absence of the *stx1* and *stx2* genes and the presence of other virulence genes *eaeA* (intimin), *ehx* (enterohemolysin), *espP* (extracellular serine protease) and *katP* (catalase-peroxidase) were confirmed by conventional PCR (primers and references see Table 2.1) by Verstraete *et al.* (2013) (Verstraete *et al.* 2013). *E. coli* O157:H7 NCTC12900 originated from a verocytotoxigenic strain which lost its ability to produce toxin. It was already used in several studies as a surrogate strain (Skandamis and Nychas 2000; Dibb-Fuller *et al.* 2001; Woodward *et al.* 2003; Vande Walle *et al.* 2011). The strains were streaked from a glycerol frozen stock maintained at −70°C onto a tryptone soy agar plate (TSA; Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h. A single colony from the plate was transferred to 25 ml of tryptone soy broth (TSB; Oxoid, Basingstoke, UK) in a 50 ml falcon tube and statically incubated at 37°C for 24 h. The bacteria were sedimented by centrifugation (5000 × g for 20 min at 4°C) and washed twice in sterile 50 mM phosphate buffered saline (PBS) (pH 7.4). Cells in the pellet were resuspended in the same amount of sterile PBS. The cell population of the inoculum was determined by plating appropriate dilutions in 0.1% peptone in duplicate on xylose lysine desoxycholate agar (XLD; Lab M, Bury, UK) for *Salmonella* and cefixime-tellurite sorbitol MacConkey agar (CT-SMAC; Lab M, Bury, UK) for *E. coli* O157:H7. Obtained plate counts were 7.9 log CFU/ml for *Salmonella* Typhimurium MB4880, 7.8 log CFU/ml for *Salmonella* Thompson RM1987, 7.7 log CFU/ml for *E. coli* O157:H7 MB3885 and 7.4 log CFU/ml for *E. coli* O157:H7 NCTC12900.

2.2.2 Seed testing

Untreated butterhead lettuce seed (*Lactuca sativa* L. var. *capitata* 'Alexandria RZ') was obtained from Rijk Zwaan Distribution B.V., De Lier, the Netherlands. Prior to inoculation, the seeds were tested in triplicate for the presence of *Salmonella* and/or *E. coli* O157:H7. Therefore, 4 ml PBS + 0.02% Tween 20 was added to 0.200 g seeds (~ 200 seeds). The samples were homogenized for 1 min at maximum speed (Homex 6, Bioreba, Reinach, Switzerland) in a sterile filter bag (Bioreba, Reinach, Switzerland). The seeds were broken down until a homogenous mixture was obtained. One hundred microliter of this mixture was plated onto both XLD and CT-SMAC in duplicate. The samples were also enriched by adding 1 ml of the homogenized mixture onto 9 ml buffered peptone water (BPW; Oxoid, Basinstoke, UK) and incubated at 37°C, 24 h, 200 rpm. The enrichment content was streaked on XLD and CT-SMAC and incubated at 37°C, 24 h.

Table 2.1: Oligonucleotide primers used to confirm the presence/absence of the EHEC virulence genes in *E. coli* O157:H7 MB3885 as described by Verstraete *et al. (Verstraete et al. 2013)*.

Primers	(5' to 3') Sequence	Target gene	Amplicon size (bp)	Reference
eaeA-F	GTGGCGAATACTGGCGAGACT	*eaeA*	890	(Desrosiers *et al.* 2001)
eaeA-R	CCCCATTCTTTTTCACCGTCG			
espP-F	GATTACAGCACGCATTCATGGTAT	*espP*	73	(Nielsen and Andersen 2003)
espP-R	TCCAGGCATCCTCAGTGACA			
katP-F	GAAGTCATATATCGCCGGTTGAA	*katP*	73	(Nielsen and Andersen 2003)
katP-R	GTCATTTCAGGAACGGTGAGATC			
vt1-F	ACACTGGATGATCTCAGTGG	*stx1*	614	(Desrosiers *et al.* 2001)
vt1-R	CTGAATCCCCCTCCATTATG			
vt2-F	GGCACTGTCTGAAACTGCTCC	*stx2*	255	(Leung *et al.* 2001)
vt2-R	TCGCCAGTTATCTGACATTCTG			

2.2.3 Seed inoculation

Inoculation of the seeds was performed at two different moments. The inoculation with *Salmonella* Typhimurium MB4880 and *E. coli* O157:H7 MB3885 was performed on September 2009 and the inoculation with the other two strains (*Salmonella* Thompson RM1987N and *E. coli* O157:H7 NTCT12900) on February 2011. For both inoculation moments, the same seed batch was used. The dry seeds (17 g) were immersed in 85 ml of the appropriate bacterial suspension (1/5 w/v) overnight (16 h) at room temperature (~ 21°C) in the biosafety hood. The excess of inoculum was removed with a 0.22 µm filter (Bottle Top Filters - 500-ml Capacity, MF75™ Series, Nalgene, USA). The seeds were

then spread thinly in sterile petri plates on a sterile filter paper and left to dry overnight (16 h) at room temperature (~ 21°C) in the airflow of a biosafety hood. Non-inoculated seeds immersed in sterile PBS were used as negative control. After drying, the seeds were stored in the dark in sterile petri plates in an incubator at 20 ± 2°C with an average relative humidity of 50 ± 7% for one year (for seeds inoculated with *Salmonella* Thompson RM1987N and *E. coli* O157:H7 NTCT12900) or two years (for seeds inoculated with *Salmonella* Typhimurium MB4880 and *E. coli* O157:H7 MB3885). The relative humidity and temperature were logged in 30-minute intervals using an EL-USB-2 data logger (Lascar Electronics).

2.2.4 Measurement of bacterial populations on the seeds

Populations of each pathogen on inoculated seeds were determined directly after inoculation and on different sampling times up to two years after inoculation. On each sampling time, three samples of 0.200 g (~ 200 seeds) were added to sterile filter bags (Bioreba) with 4 ml sterile PBS + 0.02% Tween 20 and homogenized (Homex 6, Bioreba) as described before. The resulting suspension was serially diluted in 0.1% peptone and spread-plated in duplicate onto CT-SMAC or XLD plates, respectively and incubated at 37°C for 24 h. At the end of the study, when the detection limit of direct plating method was obtained (1.3 log CFU/g seed), presence/absence testing was performed by enrichment of 1 ml of homogenized sample in 9 ml BPW incubated at 37°C, 24 h, 200 rpm. Subsequently, isolations and serological confirmation were performed as described before and hereafter, respectively.

2.2.5 Individual seed testing two years after inoculation

Two years after inoculation of the seeds with *Salmonella* Typhimurium MB4880 and *E. coli* O157:H7 MB3885 (experiment September 2009), three different methods were tested to detect the pathogens on the individual seeds. First, 25 seeds were individually placed with sterile forceps in 10 ml of BPW each, incubated for 24 h at 37°C (200 rpm) and the enrichment was streaked onto the appropriate selective media. Second, 25 seeds were individually pushed into selective media with a sterile toothpick. The plates were incubated at 37°C for 24 h and screened for presumptive growth around the seeds. Third, 25 seeds were individually pushed into TSA with a sterile toothpick. After incubation (37°C, 24 h), each seed was removed from the plate with a sterile cork bore and each TSA-seed plug was added to 10 ml of BPW (37°C, 200 rpm, 24 h). The enrichment was streaked onto the appropriate selective media. Recovery of pathogens from seeds was repeated two times within the same month (i.e. two years after inoculation).

2.2.6 Sprouting of the seeds 2 years after inoculation.

The seeds were sprouted before testing for the presence of the pathogens. Therefore, six sterile filter papers (Whatman filters N1, cut in eight pieces) were placed in a petri plate. Each filter paper was

saturated with sterile distilled water. Care was taken that the filter pieces did not touch each other. On each filter, one seed was placed with sterile forceps. The petri plates were wrapped with parafilm and placed in the dark at 20°C for three days to enable the seeds to sprout. Then they were placed in a growth chamber with a 12 h day-night cycle and a temperature of continuously 20°C. Six seedlings per inoculated seed batch were analyzed at days 3, 5, 7 and 11 after germination. For the analysis, the seed shell of the seedling was aseptically removed and was pushed into selective media with a sterile toothpick. The media were incubated at 37°C for 24 h and checked for presumptive growth around the seed shells. The seedling was placed in a sterile filter bag. Two ml of PBS + 0.02% Tween 20 was added and the sample was homogenized as described before. Appropriate dilutions in 0.1% peptone were spread-plated onto selective medium and at the same time enrichment in BPW was performed for presence/absence testing after resuscitation. Sprouting of seeds was repeated within the same month, two years after inoculation. It was repeated two times with *Salmonella* Typhimurium MB4880 and four times with *E. coli* O157:H7 MB3885.

2.2.7 Serological confirmation

To verify the presence of the inoculated pathogens, colonies isolated from the seed and seedling samples were subjected to a serological test (*E. coli* O157:H7: DR0620, Oxoid, Basingstoke; *Salmonella*: DR1108, Oxoid, Basingstoke).

2.2.8 Statistical analyses

Log-transformed data were subjected to statistical analysis software SPSS (IBM SPSS Statistics 19). A generalized negative binomial regression was used for the comparison of the reduction rates, one-way analysis of variance for all other tests. Differences between mean values were considered significant at $P < 0.05$. The linear regression equations of the survival curves were based on the log-transformed data collected during the first year and calculated using Microsoft Excel 2007.

2.3 Results and discussion

2.3.1 Long-term survival of *Salmonella* and *E. coli* O157:H7 on lettuce seeds

Prior to inoculation, neither *E. coli* O157:H7 nor *Salmonella* were detected in the used lettuce seed samples. After inoculation and drying of the seeds, the pathogen loads ranged between 7.75 to 8.63 log CFU/g seed. The survival curves of the four strains tested on lettuce seeds together with R^2 values and the linear regression equations are given in Figure 2.1. The seed batches respectively inoculated with *Salmonella* Thompson RM1987N and *E. coli* O157:H7 NCTC12900 were stored for 48 weeks, whereas the batches respectively inoculated with *Salmonella* Typhimurium MB4880 and *E. coli* O157:H7 MB3885 were stored for 104 weeks. The survival of the *Salmonella* strains on the seeds was

significantly better than the survival of the *E. coli* O157:H7 strains ($P < 0.0001$). After 48 weeks (340 days), an average decrease of 1.62 log CFU/g seed was seen for the *Salmonella* Typhimurium strain and a decrease of 1.05 log CFU/g seed for the *Salmonella* Thompson strain. The *E. coli* O157:H7 strains MB3885 and NCTC12900 decreased on average with 4.31 log and 4.70 log CFU/g seed, respectively. These decreases would correspond with reduction rates of 0.13, 0.09, 0.36 and 0.39 log CFU/g seed per month, respectively and were significantly different for *E. coli* O157:H7 and *Salmonella* ($P < 0.0001$). After 104 weeks (2 years) (data not shown), the concentration of *Salmonella* Typhimurium strain MB4880 was 7.35 ± 0.06 log CFU/g seed whereas *E. coli* O157:H7 strain MB3885 could only be detected after enrichment of the samples (< 1.3 log CFU/g seed). In general, a similar survival trend was described by Beuchat and Scouten (2002). Although they used a 1000-times lower inoculum and alfalfa seeds instead of lettuce seeds, a 1.23 log CFU/g seed reduction (25°C, a_w 40) after 52 weeks was observed for *Salmonella*, and a 3.95 log CFU/g seed reduction (25°C, a_w 0.36) after 25 weeks for *E. coli* O157:H7 after retrieval by enrichment. In the present study, higher inocula levels up to 8.63 log CFU/g seed were used to allow for quantitatively monitoring the survival of the pathogens over a longer period, as well as for recovering the pathogens after seed germination on the seedlings without the need for an excessive sample size. Studies are often criticized when using 'unrealistic' high inoculum levels, partially because inoculum effects can sometimes be seen for produce surfaces with less survival of the pathogen in case of lower inoculum levels (Richert *et al.* 2000). In this way, the behavior of the pathogens at high contamination levels could be different from their behavior at low contamination levels. However, this is not always the case. Two other studies have investigated the long-term survival of *Salmonella* on nuts, also stored under dry conditions: (i) almonds stored at 23°C ± 3°C, inoculated at levels of 8, 5, 3 and 1 log CFU/almond (Uesugi *et al.* 2006) and (ii) in-shell pecans and pecan nutmeats (Beuchat and Mann 2010) inoculated at levels of 6.94 to 6.99 and 1.85 to 1.95 log CFU/g stored at different temperatures. Both studies found comparable reduction rates when using different inoculum levels. To our knowledge, there are no reports on the survival of *E. coli* O157:H7 on seeds or nuts in which a possible inoculum effect was described. Assuming that the reduction rate of both pathogens on lettuce seeds is independent from the initial inoculum level, one could, based on the linear regression equation, estimate the time that is needed to render the pathogen undetectable per gram of seeds, for any arbitrarily chosen inoculation level. For a more realistic initial inoculum level of e.g. 1 log CFU/g seed, *Salmonella* would, following this assumption, be undetectable after approximately 36.8-38.3 weeks and *E. coli* O157:H7 after 10-11.4 weeks, depending on the strain used. This has also implications for the routine detection ability of enteric pathogens on stored seeds.

These data are based on the results of artificial inoculation experiments and it cannot be excluded that the fate of enteric pathogens naturally occurring on seeds may be different. In a 'natural' situation, the pathogen is likely been exposed to nutrient scarcity before the seed contamination event takes place (e.g. due to survival in irrigation water, dust, plant debris). So, enhanced resistance to physical or chemical challenges may occur and influence their survival on the seeds. It was already shown that the sensitivity of *Salmonella* to desiccation due to initial drying of artificially inoculated seeds was influenced by the inoculum preparation (bacteria grown on agar or in broth) (Uesugi *et al.* 2006). Therefore, it is important for future studies to try to simulate the 'natural' contamination event, or if possible, to examine the long-term survival on naturally contaminated seeds. In this way, mechanisms that possibly influence the survival of enteric pathogens on seeds, can be found.

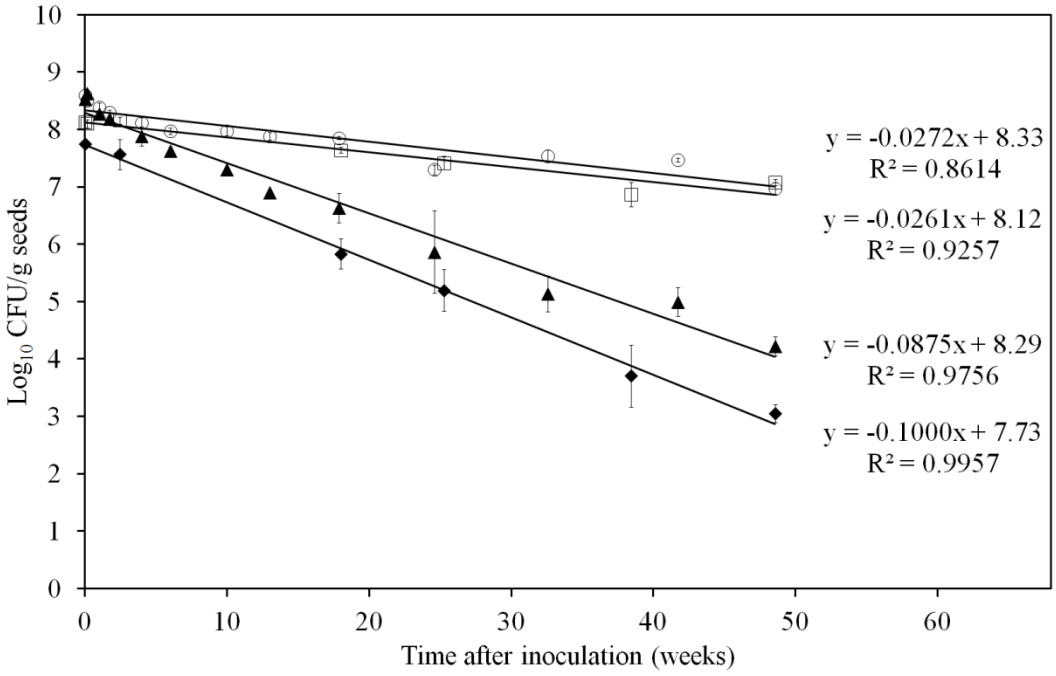


Figure 2.1: Long-term survival of *Salmonella* Typhimurium MB4880 (○), *Salmonella* Thompson RM1987N (□) and *E. coli* O157:H7 strain MB3885 (▲) and NCTC12900 (♦) inoculated at high level (± 8 log CFU/g seed) on lettuce seeds. Results shown are the averages of the counts on selective medium of three subsamples, plated in duplicate. Selective medium was XLD for *Salmonella* and CT-SMAC for *E. coli* O157:H7.

2.3.2 Individual seed testing two years after inoculation

Two years after seed inoculation, three different methods were tested for the detection of *Salmonella* Typhimurium strain MB4880 and *E. coli* O157:H7 strain MB3885 on individual seeds. In a first method, the seeds were individually placed in BPW and the enrichment was streaked onto appropriate selective media (= seed enriched in BPW). Second, seeds were individually pushed into selective medium (= seed in selective medium). And third, seeds were individually pushed into TSA and after incubation, a TSA plug with a seed was added to BPW after which the enrichment was

streaked onto appropriate selective media (= seed in TSA plug in BPW). The results are shown in Table 2.2. For *Salmonella*, all seeds tested positive with the three methods. Even directly pushing the stored seeds into selective XLD revealed the presence of *Salmonella* for every individual seed. For *E. coli* O157:H7, the highest recovery rate was obtained with the 'seed in TSA plug in BPW' method (14%), and the lowest with direct enrichment in BPW (4%), though an average recovery of 8.7% was generally obtained with all three methods. These results are in accordance with the results of Liao and Fett (2003), who have shown that for naturally *Salmonella* contaminated alfalfa seeds the use of two consecutive pre-enrichment steps led to a higher detection rate than a single pre-enrichment. The survival rate of the pathogens on the seeds was significantly different between the two pathogens with *Salmonella* demonstrating a better ability for survival on the dry seeds stored at ambient temperature.

Table 2.2: Recovery of *Salmonella* Typhimurium and *E. coli* O157:H7 from individual lettuce seeds 2 years after inoculation, using different methods of detection. Values are the sum of two experiments with 25 seeds (n=50).

Detection method		*Salmonella* Typhimurium MB4880	*E. coli* O157:H7 MB3885
seed enriched in BPW	number of positives	50/50	2/50
	% of positives	100.0	4.0
seed in selective medium	number of positives	50/50	4/50
	% of positives	100.0	8.0
seed in TSA plug in BPW	number of positives	50/50	7/50
	% of positives	100.0	14.0

2.3.3 *Salmonella* and *E. coli* 0157:H7 on seeds sprouted two years after inoculation

Details of the resuscitation and growth of the pathogens on seedlings of two-year old inoculated seeds are shown in Table 2.3. For the *Salmonella* Typhimurium strain MB4880 inoculated seeds, all 48 removed seed coats were found positive for *Salmonella* and the pathogen was able to colonize all 48 seedlings. A seedling carried on average 4.70 ± 0.50 log CFU of *Salmonella* with a maximum of 5.92 log CFU/seedling and a minimum of 3.87 log CFU/seedling. Taking into account that the initial load of *Salmonella* on the seeds before germination averaged about 7.35 log CFU/g seed, (i.e. on average 4.35 log CFU per seed) and that the seed coat was removed before the analysis of the seedling, it is most likely that *Salmonella* was able to proliferate on the seedlings. However, the numbers of *Salmonella* did not further increase three days after sprouting ($P > 0.05$). Several authors reported a similar trend for *Salmonella* on alfalfa seeds at 20-25°C where the highest *Salmonella* density was reached between one and three days after sprouting (Jaquette *et al.* 1996; Stewart *et al.*

2001b; Howard and Hutcheson 2003; Fu *et al.* 2008; Liao 2008). These data are also in accordance with Fu *et al.* (2008), who have shown that *Salmonella* could grow on alfalfa sprouts, after storage of the naturally contaminated alfalfa seeds for eight years.

For *E. coli* O157:H7 strain MB3885 inoculated seeds, 12 of the 96 seedlings (12.5%) were found positive. This 12.5% recovery rate was comparable with the detection rate on the seed (TSA plug in BPW, 14%). Apparently, a seedling was only found contaminated if the seed coat was found contaminated. It is clear from our results that *E. coli* O157:H7 was able to resuscitate and proliferate on the seedlings considering that the average amount per positive seedling was 3.74 ± 0.68 log CFU of *E. coli* O157:H7 (maximum 4.41 log CFU/seedling and minimum 2.71 log CFU/seedling), whereas samples of 200 seeds from 2-year old inoculated batches needed to be enriched for detection (< 1.3 log CFU/g seed). The average *E. coli* O157 contamination level that was found on the contaminated seedlings was approximately tenfold lower than was observed for the *Salmonella* contaminated seedlings (on average 3.74 ± 0.68 log CFU/g versus 4.70 ± 0.50 log CFU/g). This could be due to different factors: (i) the contamination level found on the seeds two years after inoculation, hence the initial inoculum level before sprouting was lower than for *Salmonella*. A positive correlation between the initial inoculum level for *Salmonella* on alfalfa seeds and the maximum population on sprouts was also reported by Liao (2008). This inoculum dependent phenomenon on sprouts is not yet described for *E. coli* O157:H7 but it is known that *E. coli* O157:H7 and *Salmonella* reach similar population densities on lettuce sprouts when seeds were inoculated with the same cell density (Jablasone *et al.* 2005). (ii) It is also possible that *E. coli* O157:H7 cells were more stressed than *Salmonella* cells as already indicated above, resulting in a slower growth rate on the sprouting seeds. This hypothesis is supported by the fact that *E. coli* O157:H7 did not reach its maximum concentration 2–3 days after sprouting, as seen for *Salmonella* in this experiment and reported in the literature for *E. coli* O157:H7 and *Salmonella* (Jaquette *et al.* 1996; Stewart *et al.* 2001a; Stewart *et al.* 2001b; Howard and Hutcheson 2003; Liao 2008). Although the highest increase was observed during the first three days after sprouting, *E. coli* O157:H7 was able to increase an extra 0.7-0.8 log CFU per positive seedling by day 11 after sprouting.

Finally, none of the enrichment samples for which no pathogens could be retrieved by means of direct selective plating, were positive. This indicates that if *Salmonella* or *E. coli* O157:H7 was present on a seed, it resuscitated within the first 3 days of sprouting and showed outgrowth to numbers of > 3 log CFU/seedling.

Table 2.3: Resuscitation and proliferation of *Salmonella* Typhimurium MB4880 and *E. coli* O157:H7 MB3885 on seedlings two years after seed inoculation.

Pathogen	Days after germination	Number of positive seed coats	Number of positive seedlings	Number of positive enrichments	Mean log CFU/positive seedling ± 1 SD[a]
Salmonella Typhimurium MB4880	3	12/12	12/12	12/12	4.72 ± 0.39
	5	12/12	12/12	12/12	4.45 ± 0.42
	7	12/12	12/12	12/12	4.71 ± 0.31
	11	12/12	12/12	12/12	4.95 ± 0.72
	Total	48/48	48/48	48/48	4.70 ± 0.50
E. coli O157:H7 MB3885	3	4/24	4/24	4/24	3.61 ± 0.76
	5	5/24	5/24	5/24	3.47 ± 0.67
	7	1/24	1/24	1/24	4.38
	11	2/24	2/24	2/24	4.34 ± 0.06
	Total	12/96	12/96	12/96	3.74 ± 0.68

[a] SD = standard deviation

2.4 Conclusions

Our study not only confirms the long-term survival of enteric pathogens on seeds (Taormina and Beuchat 1999; Beuchat and Scouten 2002) but also shows that both pathogens, after being present for two years on the seeds, maintain their ability to resuscitate and proliferate on the seedlings. Stored seeds may thus be a contamination source of enteric pathogens for leafy vegetables such as butterhead lettuce; because of their principal greenhouse cultivation, this may even be one of the major potential contamination sources. More information is needed on the hygiene level of seed production practices. On the other hand, germination of seed may be a suitable resuscitation method for routine testing of seed batches for the presence of enteric pathogens.

Chapter 3:
Survival of enteric pathogens during butterhead lettuce growth: crop stage, leaf age and irrigation

Redrafted from:

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Experimental set-up in the growth chamber with young (left) and nearly mature plants (right)

Abstract

The survival of *Salmonella enterica* serovar Thompson and *Escherichia coli* O157:H7 was investigated on growing butterhead lettuce plants in the plant growth chamber and greenhouse. All inoculation tests were made under conditions that approximate the greenhouse conditions for butterhead lettuce cultivation in Flanders (Belgium). The survival and proliferation of the pathogens on the leaves was determined at day 0, 4 and 8 after inoculation using standard plating techniques on selective medium. In the growth chamber, the extent to which both pathogens were able to multiply on the lettuce leaves was influenced by crop stage and leaf age. On young plants, the older leaves supported pathogen survival better. On nearly mature plants, pathogen population sizes were significantly higher on the old and young leaves compared with middle-aged leaves ($P < 0.001$). In the greenhouse, the environmental regime with high fluctuations in temperature and relative humidity was less conducive for the survival of *E. coli* O157:H7, though its survival on nearly mature lettuce was enhanced by overhead irrigation. The moist conditions between the folded inner leaves are likely contributing to the survival of enteric pathogens in the lettuce head. Butterhead lettuce grown in greenhouses with a sprinkle irrigation system may present a potential health hazard when contaminated near harvest. Experimental design (growth chamber versus greenhouse) largely influences enteric pathogen behavior on growing lettuce plants.

3.1 Introduction

Governments promote the consumption of fruits and vegetables but at the same time concerns have been raised about the food safety of leafy vegetables. Leafy vegetables, such as lettuce, are considered by the US Food and Drug Administration as high risk food (Klein *et al.* 2009). But also in Europe various *E. coli* O157:H7 (Welinder-Olsson *et al.* 2004; Friesema *et al.* 2008; Söderström *et al.* 2008) and *Salmonella* outbreaks (Ward *et al.* 2002; Horby *et al.* 2003; Nygård *et al.* 2008) have been related to the consumption of lettuce.

Plant surfaces may become contaminated before harvest. Irrigation water is considered a potentially important introduction route and several studies have shown its potential for the transmission of enteric pathogens to lettuce plants. However, most of these studies were conducted either under laboratory or controlled conditions in growth chambers (Brandl and Mandrell 2002; Solomon *et al.* 2003; Aruscavage *et al.* 2008; Brandl and Amundson 2008; Zhang *et al.* 2009; Aruscavage *et al.* 2010; Erickson *et al.* 2010a) or field experiments (Barker-Reid *et al.* 2009; Erickson *et al.* 2010b; Harapas *et al.* 2010; Wood *et al.* 2010; Fonseca *et al.* 2011). Few studies have compared both types of experimental setups. Furthermore, these experiments were often performed with lettuce cultivars and environmental conditions typical for the production of leafy vegetables in the United States.

This study focused on butterhead lettuce (*Lactuca sativa* L. var. *capitata*), an important leafy vegetable grown commercially in Northern European countries and regions such as Flanders (Belgium) mainly for export. The crop is cultivated in greenhouses in a continuous monoculture system using overhead sprinkler irrigation (Pauwelyn *et al.* 2011), creating conditions that, in case of contaminated irrigation water, are likely to deposit enteric pathogens on the lettuce plants. No scientific information is available concerning enteric pathogens for this lettuce cultivar in combination with its commercial growing conditions.

The aim of this study was to investigate the effects of leaf age (young, middle-aged, old), crop stage (before or after heading) and daily overhead irrigation on the survival of *E. coli* O157:H7 and *Salmonella enterica* should they be introduced by irrigation water on butterhead lettuce in Northern European greenhouses.

3.2 Material and methods

3.2.1 Strains and growth conditions

Salmonella Thompson RM1987N is a spontaneous nalidixic-acid-resistant mutant of a described clinical isolate from a patient in a cilantro-linked outbreak (Brandl *et al.* 2005b) and was kindly donated by Dr. Maria Brandl (USDA-ARS, Albany, California, USA). *E. coli* O157:H7 MB3885 naturally lacks Shiga toxin genes (*stx1* and *stx2*) and was used as a non-toxigenic surrogate strain for the Shiga toxin producing serotype O157:H7 for biosafety reasons. This strain originates from beef carpaccio and was kindly donated by the Scientific Institute for Public Health (Brussels, Belgium). The absence of *stx1* and *stx2* genes and presence of other virulence genes *eae* (intimin), *ehx* (enterohemolysin), *espP* (extracellular serine protease) and *katP* (catalase-peroxidase) were confirmed by conventional PCR (Verstraete *et al.* 2013). Both strains were streaked from a glycerol frozen stock maintained at −70°C onto a tryptone soy agar plate (TSA; Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h. One colony was transferred to 10 ml of tryptone soy broth (TSB; Oxoid, Basingstoke, UK) and incubated at 37°C for 18 h while shaken at 200 rpm.

3.2.2 Plant growth conditions

Pelletized butterhead lettuce seeds (*Lactuca sativa* L. var. *capitata* 'Alexandria') were obtained from Rijk Zwaan Distribution B.V., De Lier, the Netherlands. The seeds were sown in ground blocks of 4 x 4 x 6 cm (seed and cutting compost, Saniflor, Geraardsbergen, Belgium). Two weeks after sowing, the seedlings were placed in pots of 13 cm (for the experiments with young plants, 9-leaf stage) or 20 cm diameter (for the experiments with nearly mature plants) and grown in the greenhouse at ILVO. For

inoculations in the growth chamber, the plants were moved from the greenhouse to the growth chamber (Isocab, Harelbeke, Belgium) two days before inoculation and placed in trays with ± 2 cm irrigation water. Growth chamber conditions were set at ± 19°C during the day and at ± 12°C at night with a relative humidity (RH) of 70-80% and a photoperiod of 14 h. The greenhouse experiment was conducted in April 2010 and conditions of RH and temperature were logged in 5-minute intervals using an EL-USB-2 data logger (Lascar Electronics, Salisbury, UK) (Table 3.1).

3.2.3 Plant inoculations and irrigation

Cells of each strain were washed twice by centrifugation (6000 × g, 15 min) in 50 mM phosphate buffered saline (PBS, pH 7.4). The optical density (OD) was measured at 595 nm using a microplate reader (Bio-Rad 3550, Richmond, USA) and by using an OD - CFU/ml standard curve, the appropriate amount of cells was resuspended in PBS to give 1 x 10^5 CFU/ml. For each combination of pathogen and crop stage, a total of 14 plants were inoculated. Young plants were inoculated by immersion as described by Brandl and Amundson (2008). Nearly mature plants were spray-inoculated as immersion was not feasible for biosafety reasons. The leaves were sprayed with a hand-held sprayer until runoff, which corresponded with approximately 100 ml inoculum per plant. A preliminary test was performed to compare both inoculation methods. This test showed similar survival trends of the pathogens. The pots were wrapped with plastic film (Saran wrap; Dow chemical company) to prevent soil contamination by dripping. Both inoculation methods resulted in a pathogens level of approximately 3 x 10^3 - 10^4 CFU per gram of leaf tissue as determined by selective plating as described hereafter. The control treatment (six plants) consisted of PBS without added inoculum. To test the influence of overhead irrigation, half of the plants in each experiment were also watered from above with a hand-held sprayer until runoff, whereas the other plants were only watered from below. For all experiments, irrigation water from the experimental greenhouse at ILVO was used, which originates from groundwater. A chemical analysis of the water was performed by INAGRO (Rumbeke-Beitem, Belgium) and ILVO (Merelbeke, Belgium) (Table 3.2).

Table 3.1: Daily environmental conditions in the controlled environment growth chamber and in the greenhouse during inoculation experiments of lettuce with enteric pathogens.

Experiment	Day/ night	Temperature (°C)			Relative Humidity (%)		
		Mean ± Stdev	Min	Max	Mean ± Stdev	Min	Max
Growth chamber - young plants	day	18.5 ± 1.1	14.0	21.5	81.4 ± 6.0	64.5	93.0
	night	12.0 ± 0.9	11.0	18.0	78.0 ± 6.9	49.0	89.0
Growth chamber - mature plants	day	19.6 ± 0.7	15.5	22.0	69.9 ± 2.8	55.5	80.0
	night	12.2 ± 0.9	11.0	16.5	70.7 ± 2.9	56.5	82.0
Greenhouse - mature plants	day	21.1 ± 6.0	7.0	35.0	45.2 ± 14.4	20.0	75.5
	night	11.9 ± 2.4	7.0	21.0	65.9 ± 6.6	37.0	76.5

Table 3.2: Water characteristics of the irrigation water used for the daily overhead irrigation treatment.

pH	EC	BOD[a]	COD[b]	NO_3^-	NH_4^-	SO_4	Cl	Fe	Mn	Mg	Ca	K	Na
	µS/cm	mg/l O_2	mg/l O_2	mg/l	mg/l	mg/l	mg/l	µg/l	µg/l	mg/l	mg/l	mg/l	mg/l
7.46	637	< 5.0	< 25.0	< 5.0	< 5.0	462	148.8	20.0	40. 0	15.1	109.2	8.6	18.4

[a] biological oxygen demand
[b] chemical oxygen demand

3.2.4 Measurement of pathogen populations on lettuce leaves

Before inoculation, the leaves of young plants were labeled from old to young with small pieces of tape. Old, middle-aged, and young leaves were numbered 1-3, 4-6 and 7-9, respectively. For inoculated nearly mature lettuce heads, the fourth to sixth leaves were sampled as old leaves, the 12th to 14th leaves as middle-aged, and the leaves enclosed in the head as young leaves. Three randomly chosen plants from each treatment (i.e. pathogen – crop stage – irrigation combination) were sampled at four and eight days after inoculation, while one plant per treatment was sampled at day 0. From each plant, three leaves from each leaf age category were collected using sterile instruments. The leaves were cut approximately 1 cm above the soil surface, placed individually in a sterile extraction bag with filter (Bioreba, Reinach, Switzerland), and each leaf was weighed. Then, PBS with 0.05% v/v Tween 20 was added at 1/1 (w/v) ratio and the sample was ground for ± 15 s at maximum speed (Homex 6, Bioreba) until a homogenous mixture was obtained. Tenfold dilutions of the resulting suspension were made in 0.1% peptone and spread-plated on xylose lysine desoxycholate (XLD, LAB032; Lab M, Bury, UK) overlayed with TSA for *Salmonella* (XLD-TAL) or cefixime-tellurite sorbitol MacConkey (CT-SMAC, Lab 161; Lab M, Bury, UK) overlayed with TSA (CT-SMAC-TAL). The TAL-method was described earlier for the resuscitation of injured cells (Kang and Fung 2000; Qiu and Wu 2007). All plates were incubated at 37°C for at least 18 h. Simultaneously, a non-selective enrichment was conducted by adding 1 ml of leaf homogenate to 9 ml of buffered peptone water (BPW; Oxoid, Basingstoke, UK) and incubated at 37°C, 200 rpm for 24 h. Only, when the plate counts were below the detection limit (< 1.3 log CFU/g), 100 µl of the enrichment culture was streaked onto the appropriate selective medium.

Two different controls were performed to ensure that the inoculated strains and not the indigenous bacteria were counted. First, the undiluted leaf homogenate of the control plants was plated onto XLD-TAL and/or CT-SMAC-TAL. Second, randomly selected and presumptive non-pathogen colonies were subjected to a serological test (*E. coli* O157:H7: DR0620, Oxoid, Basingstoke; *Salmonella*: DR1108, Oxoid, Basingstoke).

3.2.5 Statistical analyses

The data were analyzed in SPSS (IBM SPSS Statistics 19) using a multi-factor analysis of variance (linear models) with $\alpha = 0.05$. The data were normalized by subtracting the mean of the log count at day 0 from the mean of the log-transformed values at days 4 and 8. The explanatory fixed variables were day (day 4 or day 8), leaf age (young – middle-aged – old) and overhead irrigation treatment (with or without). Full factorial design was first performed. If all the interaction terms were not significant, a simplified model without interaction could be used with the Scheffé-test as post-hoc test. In other instances, Fisher's Least Significant Difference (LSD) post-hoc comparisons were made with the combinations of the groups. Values below the detection limit were considered to be at the detection limit (1.3 log CFU/g) if the enrichment was positive whereas those testing negative by enrichment culture were assigned a value of 0.0 as described by Erickson *et al.* (2010a).

3.3 Results

3.3.1 Survival and growth potential on young plants in the growth chamber

The results are shown in Figure 3.1. Both pathogens were able to proliferate on lettuce leaves after inoculation. For *Salmonella*, no significant differences were found between the average counts at 4 and 8 days after inoculation ($P > 0.05$), whereas for *E. coli* O157:H7 a slight increase could be observed ($0.01 < P < 0.05$). For both pathogens, no significant differences were identified between plants with and without daily overhead irrigation, and survival on the youngest leaves was significantly lower than on the middle-aged or oldest leaves ($P < 0.001$). For *Salmonella* (Figure 3.1, A-C), the viable counts decreased by 1.14 log CFU/g on the young leaves whereas they slightly increased on the middle-aged and old leaves by 0.38 and 0.54 log CFU/g, respectively (average of days 4 and 8). For *E. coli* O157:H7 (Figure 3.1, D-F), the same trend was observed but with a greater relative increase. On average, the *E. coli* O157:H7 counts on the young leaves increased slightly (0.40 log CFU/g) and showed a greater increase on the middle-aged and old leaves (1.86 and 1.99 log CFU/g, respectively). At day 8, however, this leaf age effect was no longer measurable for the daily irrigated plants inoculated with *E. coli* O157:H7 (Figure 3.1, F). Generally, the pathogen population sizes greatly varied from one leaf to another, even between leaves of the same leaf age class.

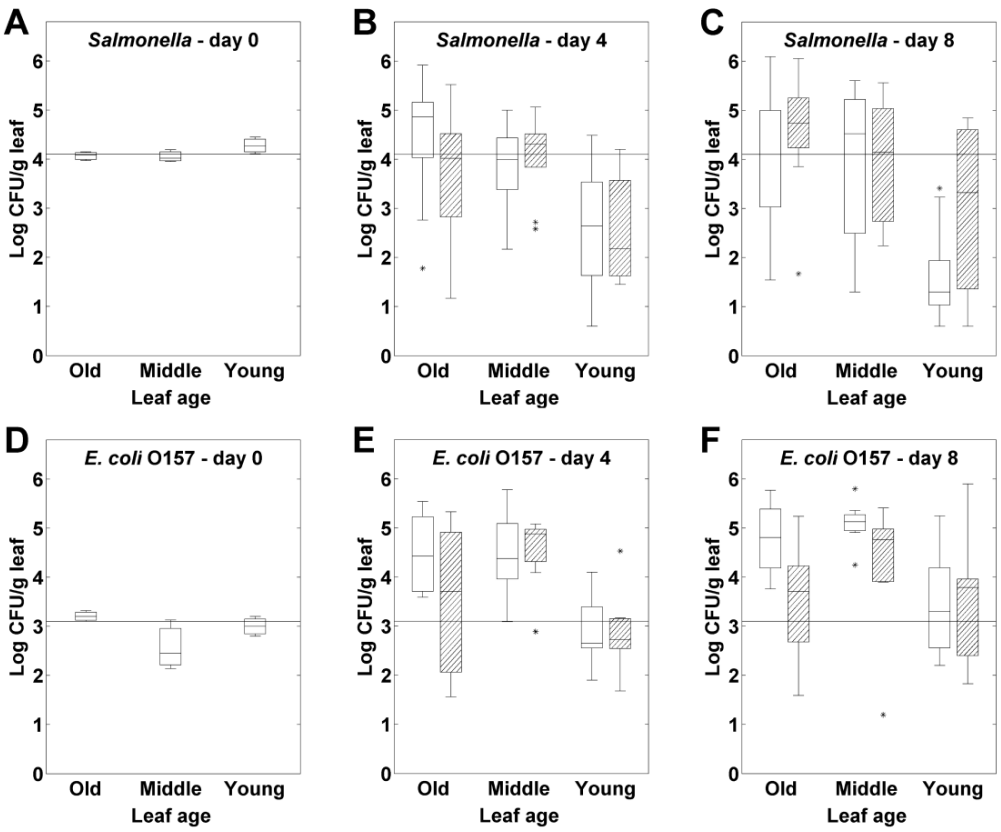


Figure 3.1: Growth chamber experiment with young butterhead lettuce (9-leaf stage): pathogen population dynamics on young, middle-aged and old leaves at 0, 4 and 8 days after inoculation. A-C, population dynamics of *Salmonella* enterica serovar Thompson strain RM1987N. D-F, population dynamics of *E. coli* O157:H7 strain MB3885. Data are presented as box plots. The non-overhead-irrigated plants are represented by white bars; the overhead-irrigated plants are represented by hatched bars. A day 0, no distinction between overhead irrigated and non-overhead-irrigated plants was made. The bottom and top of the box are the 25th and 75th percentile. The line in the box is the median and the end of the whiskers are the minimum and maximum of the data which are not outliers or extremes. Outliers and extremes are represented with an asterisk. The data are calculated from the log-transformed values of the pathogen population size per gram tissue of 6 leaf samples at day 0 and 9 leaf samples at day 4 and 8. The horizontal line shows the median of the pathogen counts at day 0.

3.3.2 Survival and growth potential on nearly mature plants in the growth chamber

The results are shown in Figure 3.2 (A-B, *Salmonella*) and Figure 3.3 (A-C, *E. coli* O157). Leaf age had a significant effect ($P < 0.001$) and overhead irrigation had no effect. Leaf age did not have the same effect for nearly mature plants as compared to the young plants: *E. coli* O157:H7 proliferated on young inner leaves and oldest outer leaves (an average increase of 0.67 and 0.28 log CFU/g, respectively) and decreased on the middle-aged lettuce leaves (on average -0.76 log CFU/g). This leaf age effect was most obvious on day 4 and was no longer observed on day 8 after inoculation. On average, the total number of pathogens per plant did not increase significantly during the 8 days in the growth chamber. Similar trends were also observed for *Salmonella,* though this experiment was only conducted until day 4 (Figure 3.2 A-B).

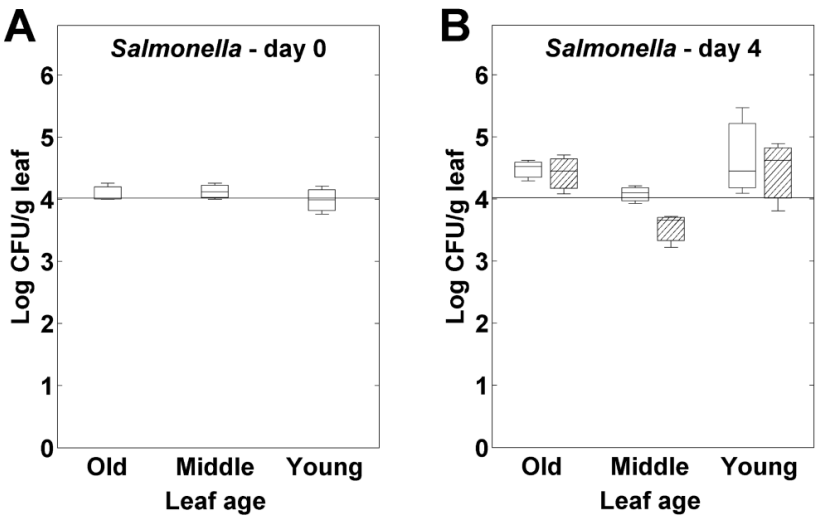


Figure 3.2: Growth chamber experiment with nearly mature butterhead lettuce: population dynamics of *Salmonella enterica* serovar Thompson strain RM1987N on young, middle-aged and old leaves at day 0 day (A) and day 4 (B) after inoculation in the growth chamber. Data are presented as box plots. The non-overhead-irrigated plants are represented by white bars; the overhead-irrigated plants are represented by hatched bars. A day 0, no distinction between overhead irrigated and non-overhead-irrigated plants was made. The bottom and top of the box are the 25th and 75th percentile. The line in the box is the median and the end of the whiskers are the minimum and maximum of the data which are not an outlier or extreme. The data are calculated from the log-transformed values of the pathogen population size per gram tissue of 6 leaf samples at day 0 and 9 leaf samples at day 4. The horizontal line shows the median of the pathogen counts at day 0.

3.3.3 Survival and growth potential on nearly mature lettuce in the greenhouse

The results are shown in Figure 3.3 (D-F). On average no increase in pathogen counts was observed and the survival of *E. coli* O157:H7 was significantly lower than in the experiment conducted in the growth chamber. On day 4, *E. coli* O157:H7 survived the best (initial inoculum density was retained) on young, overhead-irrigated leaves ($P < 0.01$). Only at day 4, a leaf age effect existed between young and middle-aged leaves of the overhead-irrigated plants. On day 8, the *E. coli* O157:H7 level was significantly higher on old leaves of overhead-irrigated plants, whereas an enrichment step was needed to detect the pathogen on old leaves of non-irrigated heads ($0.01 < P < 0.05$). On the middle-aged leaves, no statistically significant influence of overhead irrigation was observed. The average number of *E. coli* O157:H7 had decreased to 1.54 ± 0.88 log CFU/g for the daily overhead-irrigated plants, and to 1.38 ± 0.38 log CFU/g leaf for the non-irrigated plants. In total 13 of the 54 samples had to be enriched but in 10 of these 13 samples the pathogen could still be detected. This experiment could not be performed for *Salmonella* for biosafety reasons.

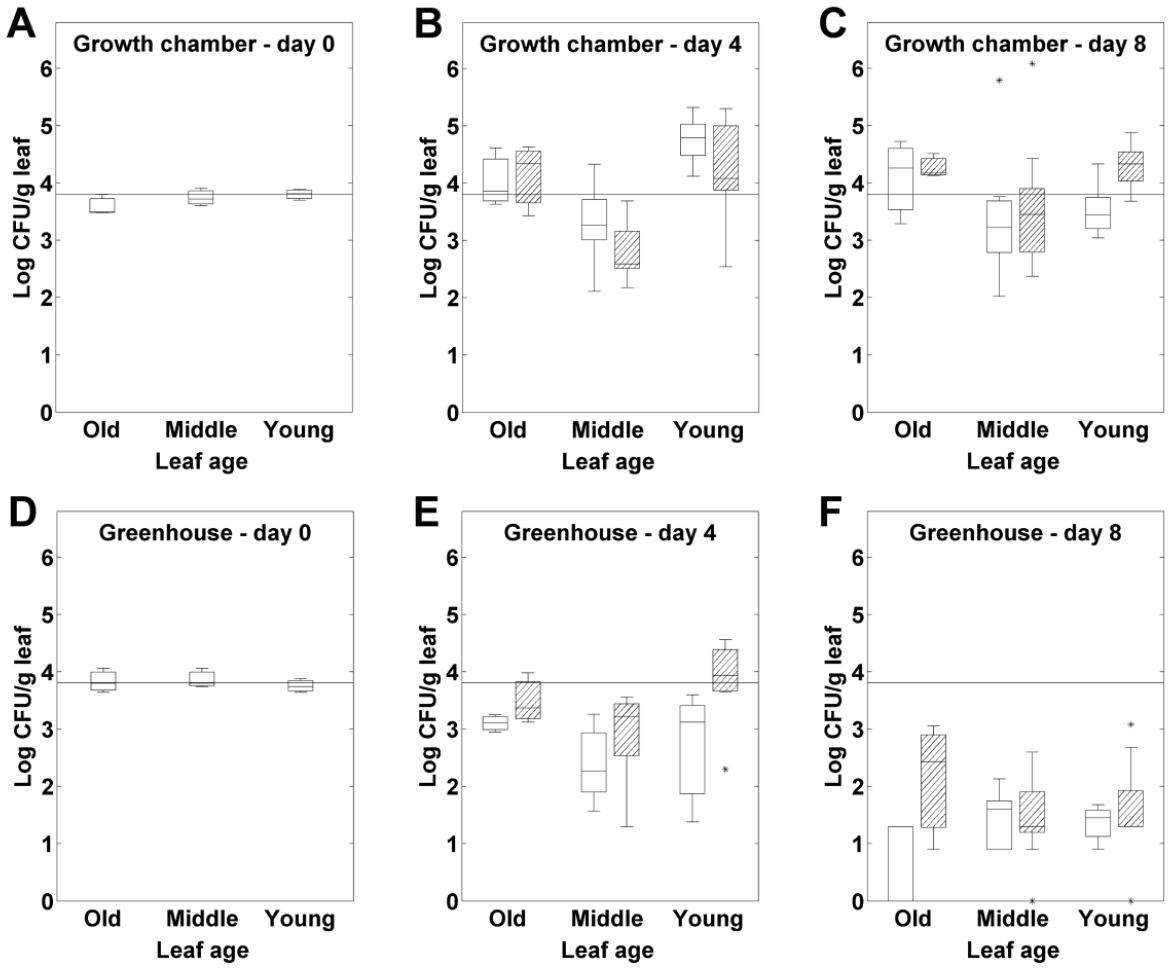


Figure 3.3: Preharvest population dynamics of *E. coli* O157:H7 strain MB3885 on young, middle-aged and old leaves of nearly mature butterhead lettuce plants at 0, 4 and 8 days after inoculation in the growth chamber and in the greenhouse. A-C, results from the growth chamber experiment. D-F, results from the greenhouse experiment. Data are presented as box plots. The non-overhead-irrigated plants are represented by white bars; the overhead-irrigated plants are represented by hatched bars. A day 0, no distinction between overhead irrigated and non-overhead-irrigated plants was made. The bottom and top of the box are the 25th and 75th percentile. The line in the box is the median and the end of the whiskers are the minimum and maximum of the data which are not outliers or extremes. Outliers and extremes are represented with an asterisk. The data are calculated from the log-transformed values of the pathogen population size per gram tissue of 6 leaf samples at day 0 and 9 leaf samples at day 4 and 8. The horizontal line shows the median of the pathogen counts at day 0.

3.4 Discussion

In the present study, we looked at plant related factors such as leaf age, crop stage and overhead irrigation to see whether these can have a potential influence on the survival of enteric pathogens in the phyllosphere of lettuce. In comparison with other studies on the survival of these pathogens on fresh produce (Aruscavage *et al.* 2008; Brandl and Amundson 2008; Erickson *et al.* 2010a), we used growing butterhead lettuce plants. The experiments were performed under the controlled conditions

of a growth chamber, but in parallel an experiment in the greenhouse was made to evaluate whether the investigated factors are also important under conditions of current practice.

The experiments in the growth chamber revealed that lettuce leaf age in combination with crop stage has an effect on the survival capacity of the pathogen. The first evidence for this phenomenon was presented by Brandl and Amundson (2008). The experimental design of our growth chamber study was quite similar to theirs, but differed in the used lettuce type (butterhead lettuce with softer folded leaves and closed head formation versus romaine lettuce with upright long leaves and semi-open head formation), lower temperatures (19°C day – 12°C night vs. constantly 28°C), lower RH (70-80% vs. 100%) and, longer sampling period (8 days vs. 2-3 days). For nearly mature plants, the pathogen population dynamics on the leaves at day 4 after inoculation were in agreement with their findings. The highest population size of *E. coli* O157:H7 found on the young inner leaves, which are preferably consumed, was on average 1.4 log CFU/g higher than on the middle-aged leaves. However, at day 8 after inoculation, when the plants were ready for harvest, this young-leaf effect was no longer observed. Brandl and Amundson (2008) reported for young plants as well as for nearly mature plants a similar young-leaf effect. In contrast, the population size of the pathogens on young plants in our study was on average 1.60 log CFU/g lower compared to the old and middle-aged leaves. Our results do not necessarily contradict the findings of Brandl and Amundson (2008), as we may have missed the young leaf effect on young plants because of our later sampling of day 4 after inoculation. This was as well suggested by an additional experiment that included a sampling at day 1 after inoculation and whereby growth of the pathogen was noted after one day (data not shown). Several factors may explain our different results on young leaves of young plants. During the 8 days in the growth chamber, the young leaves became more mature and the nutrient availability may have changed, furthermore, changes in the secondary metabolite production may have occurred. Brandl and Amundson ascribed the leaf-age dependent growth to the different nitrogen content in the exudates from leaves of different ages. The bigger relative change in leaf size of young leaves of young plants may also have an effect on the pathogen counts. As an illustration, the average weight of the young leaves increased with ± 36% whereas the old and middle-aged leaves of the young plants did not grow much. Also, it is likely that in contrast to the folded young leaves of nearly mature lettuce, the younger leaves of young plants are more susceptible to desiccation over time because they lack protection from other leaves. The observed leaf-age dependent trend may be typical for bacteria-plant interaction as Brandl *et al.* (2008) described similar survival/growth trends for the indigenous lettuce bacteria and enteric pathogens.

Several authors have highlighted the importance of the relative humidity (RH) conditions for survival of bacterial pathogens on plants (O'Brien and Lindow 1989; Brandl and Mandrell 2002; Stine *et al.*

2005). It was shown that *Salmonella* could barely grow on plants that were kept below 40-50% RH but that short periods of high RH were sufficient to recover maximum population size on the leaves (Brandl and Mandrell 2002). Therefore, we expected that daily overhead irrigation, hence higher humidity, could lead to an increased growth of the pathogens on the leaves. However, in the growth chamber additional daily overhead irrigation on top of the relative high humidity (70-80%) had no effect on the behavior of the pathogens. In the greenhouse, the effect of overhead irrigation was only sporadic. The RH was 45% on average during the day with higher values up to 76% measured at night, but this did not seem to be sufficient for resuscitation of *E. coli* O157:H7 on the leaves. The plants were daily irrigated in the morning but after two hours most of the water on the leaves was already evaporated, therefore, the period of higher relative humidity may not have been long enough. If pathogens are internalized into the leaf tissue, the irrigation treatment may have no effect as well. This could not be investigated as the used set-up of our study did not allow for making a distinction between endo- and epiphytical pathogen populations.

Furthermore, large fluctuations in temperature were measured in the greenhouse. The influence of such fluctuations has not been intensively studied. For natural substrates, Semenov *et al.* (2007) showed that *E. coli* O157:H7 and *Salmonella* inoculated in cow manure were very sensitive to fluctuating temperatures (Semenov *et al.* 2007). Future research should be directed to investigate the role of environmental fluctuations on plant-pathogen interactions in order to gain better insights in the fitness of enteric pathogens on plants grown under commercial conditions.

3.5 Conclusion

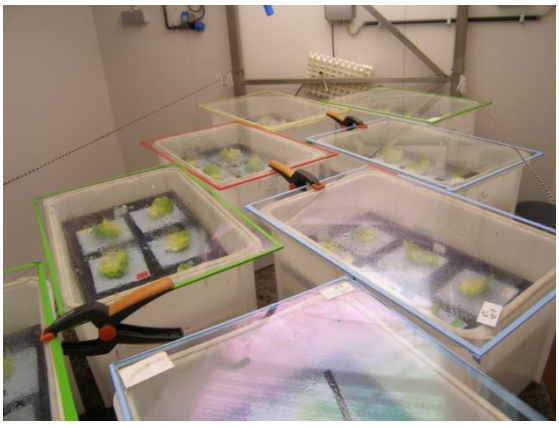
In this study it was demonstrated that leaf age, crop stage, and experimental design can have an important influence on the survival and proliferation of *E. coli* O157:H7 and *Salmonella* on butterhead lettuce. Greenhouse conditions with high fluctuations in temperature and RH, were less favorable for enteric pathogen growth compared to controlled conditions. The impact of daily overhead irrigation was only observed in the greenhouse on nearly mature plants, indicating that sprinkler irrigation may increase the food safety risk, especially after lettuce heading.

Chapter 4:

Enteric pathogen survival varies substantially in irrigation water from Belgian lettuce producers

A version of this chapter is accepted for publication in International Journal of Environmental Resources and Public Health

Van der Linden, I., Cottyn, B., Uyttendaele, M., Berkvens, N., Vlaemynck, G., Heyndrickx, M. & Maes, M.



Experimental set-up of the lettuce experiment

Abstract

It is accepted that irrigation water is a potential carrier of enteric pathogens such as *Salmonella* and *E. coli* O157:H7 and, therefore, a source for contamination of fresh produce. We tested this by comparing irrigation water samples taken from five different greenhouses in Belgium. The water samples were inoculated with four zoonotic strains, two *Salmonella* and two *E. coli* O157:H7 strains, and pathogen survival and growth in the water was monitored up till 14 days. The influence of water temperature and chemical water quality was evaluated and the survival tests were also performed in water samples from which the resident aquatic microbiota had previously been eliminated by filter sterilization. Pathogen's survival differed greatly in the different irrigation waters. Three water samples contained nutrients to support important growth of the pathogens, another enabled weaker growth, but for all, growth was only observed in the samples that did not contain the resident aquatic microbiota. In the original waters with their specific water biota, pathogen levels declined. The same survival tendencies existed in water of 4°C and 20°C, although always more expressed at 20°C. Low water temperatures resulted in longer pathogen survival. Remarkably, the survival capacity of two *E. coli* 0157:H7 strains differed, while *Salmonella* Thompson and *Salmonella* Typhimurium behaved similarly. The pathogens were also transferred to detached lettuce leaves while suspended in two of the water samples or in a buffer. The effect of the water sample on the pathogen's fitness was also reproduced on the leaves when stored at 100% relative humidity. Inoculation of the suspension in buffer or in one of the water samples enabled epiphytic growth and survival, while the pathogen level in the other water sample decreased once loaded on the leaves. Our results show that irrigation waters from different origin may have a different capacity to transmit enteric pathogens and an important impact on the fitness of the pathogens to sustain and even grow on the leaf surface.

4.1 Introduction

Salmonella enterica and *Escherichia coli* O157:H7 (*E. coli* O157:H7) are the two most important bacterial pathogens associated with foodborne illness caused by the consumption of fresh produce (Sivapalasingam *et al.* 2004). Lettuce is the single most implicated commodity (Rangel *et al.* 2005). Fresh produce may become contaminated at every stage of the production process. But before harvest, irrigation water is considered an important introduction route (Nygård *et al.* 2008; Söderström *et al.* 2008; Gu *et al.* 2011; Cevallos-Cevallos *et al.* 2012). Groundwater may become contaminated by leaching of material through the soil, originating from e.g. organic manure or feces from adjacent fields, whereas pond water may also directly become contaminated by fecal deposition (Steele and Odumeru 2004; Nygård *et al.* 2008; Söderström *et al.* 2008; Semenov *et al.*

2009; Gu *et al.* 2012). These different water sources are used for irrigation by Belgian growers that produce lettuce in greenhouses. There is, however, a lack of information to which extent the risk for contamination of the plant products is comparable between different situations (Holvoet 2014). In the present research, the survival capacity of the pathogens in irrigation water samples from five Belgian lettuce producing sites was investigated. These waters were characterized and stored at 4°C at 4°C (± average minimum winter temperature Belgian surface water) and 20°C (± average maximum summer temperature Belgian surface water) (Van Vliet and Zwolsman 2008; Anibas *et al.* 2011), with and without addition of four enteric bacterial strains, two *Salmonella* and two *Escherichia coli* O157:H7 strains. Interaction of the pathogens with the resident aquatic biota and the influence of the chemical water quality was studied by comparing the survival of the pathogenic strains in previously filter sterilized and untreated water. Pathogen survival was compared in these water conditions, but also afterwards when transmitted to leaves, which was tested in a lab scale experiment.

4.2 Material and methods

4.2.1 Strains and growth conditions

For the survival experiments in irrigation water, four pathogen strains were used. *Salmonella* Thompson RM1987N, a spontaneous nalidixic acid-resistant mutant of *Salmonella* Thompson strain RM1987, was kindly donated by Dr. Maria Brandl (USDA-ARS, Albany, California, USA). Strain RM1987 is a previously described clinical isolate from a patient in a cilantro-linked outbreak in California (Brandl and Mandrell 2002). *Salmonella* Typhimurium PT 120/ad MB4880 (MB collection of the molecular bacteriology lab of ILVO-Technology & Food Science Unit, Melle, Belgium) was isolated from overshoes at a pig farm in Belgium. *E. coli* O157:H7 MB3885 was isolated from beef carpaccio and kindly donated by the Scientific Institute for Public Health (Brussels, Belgium) and *E. coli* O157:H7 NCTC12900 by Dr. Martin Woodward (Department of Bacteriology, VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom). Both *E. coli* O157:H7 isolates lack Shiga toxin genes (*stx1* and *stx2*) and were used for biosafety reasons as non-toxigenic surrogate strains for the Shiga toxin producing (STEC) serotype O157:H7. For *E. coli* O157:H7 MB3885, the absence of the *stx1* and *stx2* genes and the presence of other virulence genes *eaeA* (intimin), *ehx* (enterohemolysin), *espP* (extracellular serine protease) and *katP* (catalase-peroxidase) were confirmed by conventional PCR as previously described (Verstraete *et al.* 2013). *E. coli* O157:H7 NCTC12900 originated from a verocytotoxigenic strain which lost its ability to produce toxin. It was already used in several studies as a surrogate strain (Skandamis and Nychas 2000; Dibb-Fuller *et al.* 2001; Woodward *et al.* 2003; Vande Walle *et al.* 2011).

For the experiments with artificially inoculated lettuce, green fluorescent protein (GFP) transformed strains were used: *Salmonella* Thompson RM1987N (Brandl *et al.* 2005b) and *E. coli* O157:H7 MB3885 (own constructs, plasmid pGFP, Clonetech, California, USA). GFP labeled strains were used to be able to distinguish the pathogens from the resident aquatic microbiota on the lettuce as previously described (a.o. (Brandl *et al.* 2005b; Franz *et al.* 2007a; Franz *et al.* 2007b; Semenov *et al.* 2010)).

All strains were taken from a glycerol frozen stock maintained at −70°C, streaked onto a tryptone soy agar plate (TSA; Oxoid, Basingstoke, UK), and incubated at 37°C for 24 h. A single colony from the plate was transferred to 10 ml of tryptone soy broth (TSB; Oxoid, Basingstoke, UK) and incubated at 37°C for 20 h at 200 rpm. The appropriate antibiotic was added to these media when GFP labeled strains were used. This was 15 µg/ml gentamicin (G1264, Sigma-Aldrich, S. Louis, MO, USA) for *Salmonella* Thompson RM1987N GFP and 50 µg/ml ampicillin (G9518, Sigma-Aldrich) for *E. coli* O157:H7 MB3885 GFP.

The stability of the GFP plasmid in the bacteria was examined by tracing the GFP expression as previously described (Lapidot and Yaron 2009) but with a few modifications. The GFP labeled bacteria were inoculated into TSB broth without antibiotics. Samples of the cultures were diluted (1:1000) in fresh medium daily, incubated for 24 h, 200 rpm, 37°C, and transferred again. Plate counting on TSA plates with or without antibiotic was performed daily to quantify the functional stability of the plasmid. The fluorescence of the colonies was checked under UV light (366 nm). Non-fluorescent colonies and randomly selected fluorescent colonies were streaked onto the appropriate selective medium. This was xylose lysine desoxycholate agar (XLD; LAB032; Lab M, Bury, UK) for *Salmonella* and cefixime-tellurite sorbitol MacConkey agar (CT-SMAC; Lab 161; Lab M, Bury, UK) for *E. coli* O157:H7. The plates were incubated at 37°C for 24 h.

4.2.2 Irrigation water samples

Irrigation water was collected (ten-liter samples) at four commercial greenhouses in Belgium where lettuce is grown and at the greenhouse complex of ILVO. Three groundwater (GW1-3) samples and two pond water (PW1-2) samples were sterilely taken.

These water samples (untreated) were stored for maximum 24 h at 4°C before the start of the experiment. Parallel to this, subsamples were sterilized by passing through a 0.22 µm filter (Bottle Top Filters - 500-ml Capacity, MF75™ Series, Nalgene, USA), and stored immediately at -18°C until used. Another set of subsamples (1 l) was analyzed by INAGRO (Rumbeke-Beitem, Belgium) and ILVO (Merelbeke, Belgium) for the following parameters: electrical conductivity (EC), pH, and concentrations of Cl, SO_4, NO_3, NO_2, NH_4, Na, K, Ca, Mg, Fe, Mn and Zn. The chemical characteristics

of the water samples are presented in Table 4.1. The biological oxygen demand (BOD) and chemical oxygen demand (COD) were measured for PW1 and GW1 but the values were below detection (< 5 mg/l O_2 resp. < 25 mg/l O_2) The water samples were checked for presence of *Salmonella* and *E. coli* O157:H7. Therefore, three times 1 ml was enriched in 9 ml buffered peptone water (BPW; Oxoid, Basingstoke, UK) and incubated at 37°C, 24 h, 200 rpm. These enrichments were then streaked onto the selective media XLD and CT-SMAC. The plates were incubated at 37°C for 24 h and checked for presumptive colonies. Presumptive *Salmonella*-type colonies were observed in GW1 and PW1 but the colonies were negative for *Salmonella* by serological testing (DR1108, Oxoid, Basingstoke).

Table 4.1: Chemical water characteristics of the five irrigation water samples.

	pH-H_2O	EC	NO_3^-	NH_4^-	SO_4	Cl	Fe	Mn	Mg	Ca	K	Na	Cu	Zn	NO_2
		µS/cm	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	µg/l	mg/l	mg/l
GW1[a]	7.45	710	0.383	< 0.22	78.64	51.2	0.52	0.15	15.60	94.30	19.00	29.40	< 0.01	< 0.01	< 0.12
GW2[a]	6.99	1413.0	198.5	< 0.22	340.9	48.8	0.08	0.06	43.50	219.90	58.85	38.00	0.03	0.73	< 0.12
GW3[a]	7.56	698.0	0.9	< 0.3	134.4	42.4	0.02	< 0.01	15.00	102.61	8.25	12.72	< 0.01	0.79	< 0.3
PW1[b]	6.91	106	0.278	< 0.22	7.28	7.6	0.14	0.06	2.37	8.77	6.75	4.18	0.01	< 0.01	< 0.12
PW2[b]	7.71	651.0	5.2	< 0.22	165.5	46.3	0.02	0.02	14.80	82.65	13.20	29.70	< 0.01	0.01	< 0.12

[a] GW = groundwater
[b] PW = pond water

4.2.3 Pathogen inoculation of irrigation water samples

An overview of the different experimental conditions and used strains is shown in Table 4.2. Freshly grown strains were washed twice by centrifugation (13 000 × g, 1 min) and resuspending the pellet in distilled water. The optical density (595 nm) of the cultures was measured and the appropriate amount of bacterial suspension was added to 720 ml of irrigation water in order to obtain a pathogen concentration of approximately 3.5 log CFU/ml. For each experimental condition, twelve sterile loosely capped vials (microcosms, Sigma-Aldrich, 60 ml) were filled with 20 ml of inoculated water. The vials were statically placed in the dark in a constant temperature of 20°C or 4°C. Uninoculated water samples (both untreated and filter sterilized) stored at 20°C and 4°C were used as negative controls.

4.2.4 Quantification of pathogen survival in the irrigation water samples

Directly after inoculation and 2, 6 and 14 days thereafter, three replicate vials were randomly taken from each experimental condition. They were vortexed on maximum speed for 15 s and the pathogen level was determined by plating dilutions (in 0.1 % peptone) onto the corresponding

selective media XLD or CT-SMAC incubated for 24 h at 37°C and on the non-selective medium TSA incubated for 24 h at 42°C. The choice to plate on TSA incubated at 42°C was based on the fact that for two water samples (GW1 & PW1), plating on selective medium was impossible due to the

Table 4.2: Overview of the 4 pathogen strains and 14 variables tested to measure pathogen survival and resident heterotrophic bacteria in each irrigation water sample.

Water treatment	Inoculation of the water with					Storage temperature of inoculated water
	Salmonella Thypimurium MB3885	*Salmonella* Thompson RM1987N	*E. coli* O157:H7 MB3885	*E. coli* O157:H7 NCTC12900	Non inoculated	
untreated		x	x		x	4°C
filter sterilized		x	x		x	4°C
untreated	x	x	x	x	x	20°C
filter sterilized		x	x		x	20°C

presence of interfering non-*Salmonella* black colonies on the XLD-plates. A preliminary test had shown that the plate counts on TSA incubated at 42°C were not significantly different from these incubated at 37°C while the growth of the natural microbiota was strongly reduced at 42°C. Two different controls were performed on the TSA-plates. First, there were no plate counts with the non-inoculated irrigation water samples, and secondly, randomly selected colonies grown on TSA reacted correctly in serological tests for *E. coli* 0157:H7 (DR0620, Oxoid, Basingstoke) and *Salmonella* (DR1108, Oxoid, Basingstoke). The limit of detection by plating was 0.6 log CFU/ml and achieved by plating 0.25 ml on a plate. Samples negative by plating were subjected to an enrichment step in BPW, therefore, 1 ml of the sample was added to 9 ml BPW as described above. For the calculations, samples positive after enrichment were considered to be at the detection limit of plating (0.6 log CFU/ml), samples testing negative after enrichment were assigned a value of 0.0 as described by Erickson *et al.* (Erickson *et al.* 2010a).

4.2.5 Quantification of the resident heterotrophic bacteria in the irrigation water samples

For the untreated samples, the heterotrophic count was determined by plating tenfold dilutions onto water plate count agar (WPCA, 6 g/l tryptone, 15 g/l bacto agar, 3 g/l yeast extract) and incubated for 5 days at 20°C. This was done for both the water samples inoculated with a pathogenic strain and for

the negative controls. In the first case, the pathogen counts were subtracted from the WPCA counts as described previously (Brandl and Amundson 2008).

4.2.6 Plant growth conditions

Pelletized butterhead lettuce seed (*Lactuca sativa* L. var. *capitata* 'Alexandria RZ') was obtained from Rijk Zwaan Distribution B.V., De Lier, the Netherlands. The plants were grown in the ILVO greenhouse in commercial potting soil (seed and cutting compost, Saniflor, Geraardsbergen, Belgium), in pots of 20 cm diameter till fully headed, mature plants (approximately 16 weeks).

4.2.7 Pathogen inoculation of lettuce leaves

Bacterial inocula were prepared in five hundred ml of untreated groundwater sample 3 (GW3), untreated pond water sample 2 (PW2) or phosphate-saline buffer (PBS) (50 mM, pH 7.4) to which GFP labelled *Salmonella* Thompson or *E. coli* O157:H7 MB3885 (in the same concentrations) were added. These suspensions were then immediately applied on plant leaves. Young inner leaves of mature lettuce crops were cut approximately 1 cm above the soil surface. For each test combination, 9 detached leaves were dipped for 3 seconds in the appropriate suspension, allowed to drip off. The initial contamination levels were ± 3.5 log CFU/g leaf (see also 4.2.8). The leaves were then placed in trays with paper towel (random design), allowed to dry (30 min) in the biosafety cabinet and subsequently transferred into plastic boxes that had a 10 cm layer of water in the bottom. The boxes were closed with glass plates to reach 100% relative humidity (see picture front page Chapter 4) and placed in a growth chamber with a 14/10 h day/night regime at 20°C/12°C. Relative humidity and temperature in the boxes were logged every 5 minutes with an EL-USB-2 data logger (Lascar Electronics). Pathogen levels on the leaves were followed for three days.

4.2.8 Quantification of pathogen survival on the lettuce leaves

Thirty minutes (day 0), and 1 and 3 days after inoculation, three leaves of each test combination were randomly selected, individually placed in a sterile extraction bag with filter (Bioreba, Reinach, Switzerland), and weighed. After addition of PBS with Tween 20 (0.05% v/v) at a 1/1 (w/v) ratio, the leaves were ground for 15 s at maximum speed with a Homex 6 (Bioreba), which generated a homogenous mixture. Tenfold dilutions (in 0.1% peptone) of the extracts were plated on TSA supplemented with the appropriate plasmid encoded antibiotic. The plates were incubated at 37°C for 24 h and the fluorescent colonies were counted under UV light (366 nm).

4.2.9 Statistical analysis

The pathogen survival experiments in irrigation water were performed one time, three vials were analyzed at each time point for each investigated condition.. A Kruskal Wallis (IBM SPSS Statistics 19)

test was performed to determine the overall effects of the plating medium (selective medium XLD or CT-SMAC vs. TSA 42°C), filter sterilization treatment and water sample. The influence of the plating medium was analyzed using a full factorial negative binomial regression with day and plating medium integrated as factors in the regression model for each water sample, strain, filtering treatment and temperature combination. The influence of the different treatments on the survival of the pathogens in the irrigation water was analyzed by means of a full factorial negative binomial regression with day, temperature and filtering treatment integrated as factors in the regression model for each water sample and strain combination. The difference in survival between the different strains was analyzed using a full factorial negative binomial regression with day and strain as factors for the untreated water samples stored at 20°C. Each analysis with the negative binomial regression models started with a saturated model and interactions and non-significant main factors were sequentially dropped at a significance level of 0.05. The most parsimonious model was used when analysing the data. Negative binomial regression was performed using the GENMOD procedure in SAS 9.4. The experiments with lettuce leaves were performed twice with three lettuce leave samples on different days and with lettuce heads grown different moments. The results were analyzed by day. A non-parametric Kruskall-Wallis test with pairwise comparison and taking the necessary Bonferoni corrections into account, was performed (IBM SPSS Statistics 19).

4.3 Results

4.3.1 Pathogen's survival in irrigation water; influence of water temperature and resident aquatic biota.

Figure 4.1 (A-F) and Figure 4.2 (A-D) show the survival of *Salmonella* Thompson (ST) and *E. coli* O157:H7 MB3885 (EC) in the five irrigation water samples. The results from the TSA platings at 42°C are shown. In general, higher populations of pathogens were recovered from the TSA platings compared with the respective selective media (CT-SMAC and XLD). This effect was more often observed in water samples stored at 4°C (14/15) in comparison with at 20°C (5/15) (see http://studwww.ugent.be/~ivdlinde/Supplemental_information_survival.html). Except for GW3, higher pathogen counts were observed at 20°C in filter sterilized water in comparison with untreated water and these differences were significant for GW1, PW1 and PW2 (for GW1,PW1 and PW2, EC and ST, $P<0.0001$). The biggest differences between filter sterilization treatment or not were observed in PW1, in this water sample both pathogens survived significantly better at 4 and 20°C (ST 4°C, ST 20°C and EC 20°C, $P<0.0001$; EC 4°C, $P<0.001$) in filter sterilized water in comparison with the untreated samples stored at the same temperature. The highest pathogen counts were detected in water samples that were filter sterilized and stored at 20°C as well. Moreover, in three of these

water samples both pathogens were able to grow within the first six days after inoculation (up to 6.03 log/ml) (GW1, PW1 and PW2). This was not noticed at 4°C. Both pathogens survived in general better in untreated water kept at 4°C instead of 20°C, growth was never observed in untreated water.

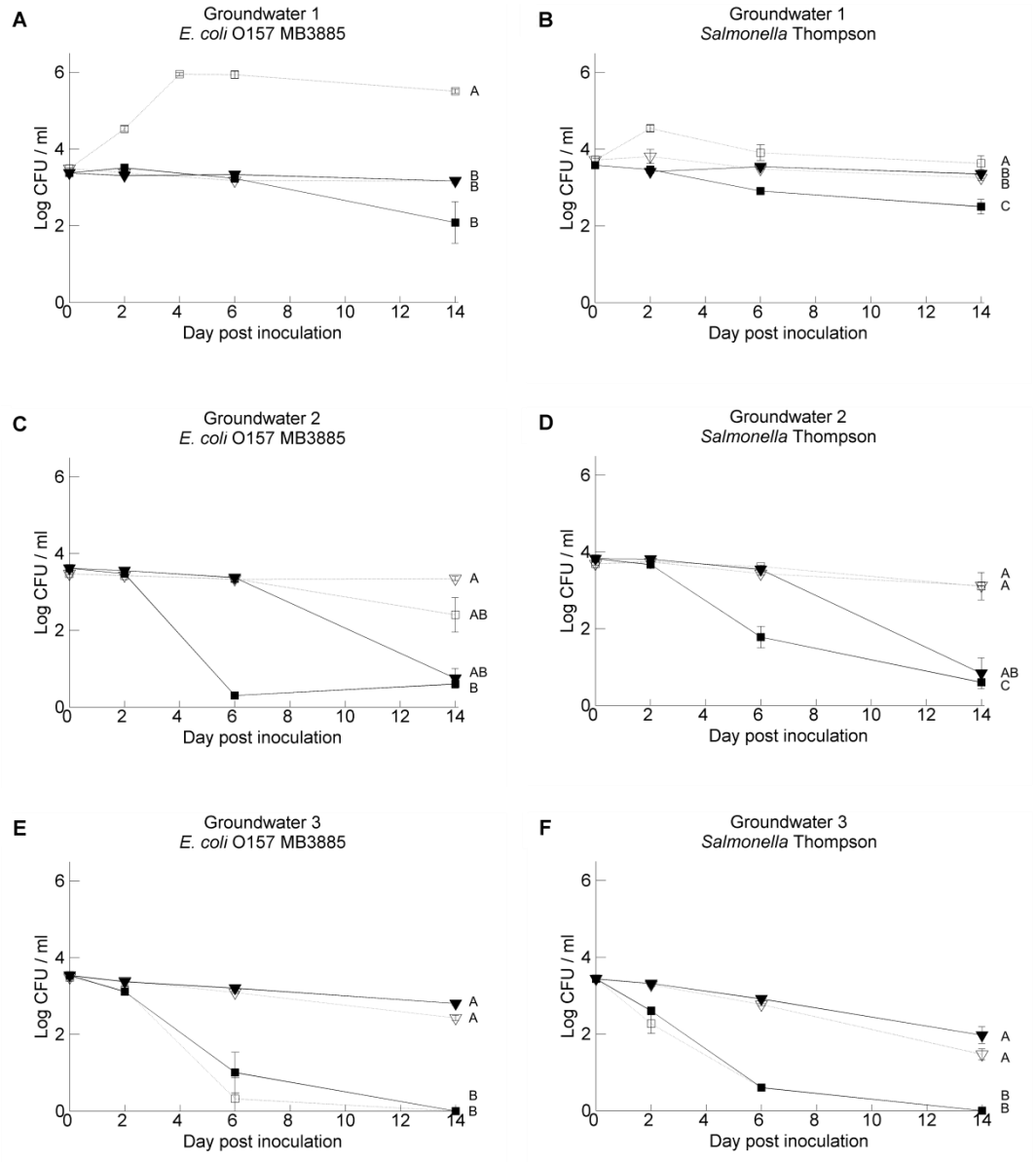


Figure 4.1: Survival of *E. coli* O157:H7 MB3885 (left) and *Salmonella* Thompson RM1987N (right) in 3 groundwater samples with the following treatments: untreated water samples stored at 4°C (full line, ▼), filter sterilized water samples stored at 4°C (dashed line, ▽), untreated water samples stored at 20°C (full line, ■), filter sterilized water samples stored at 20°C (dashed line, □). The data show the mean of three analyzed vials and are calculated from the log transformed values of the pathogen population size. Error bars indicate standard deviations. Different letters indicate significant difference ($P < 0.05$) between means according to negative binomial regression.

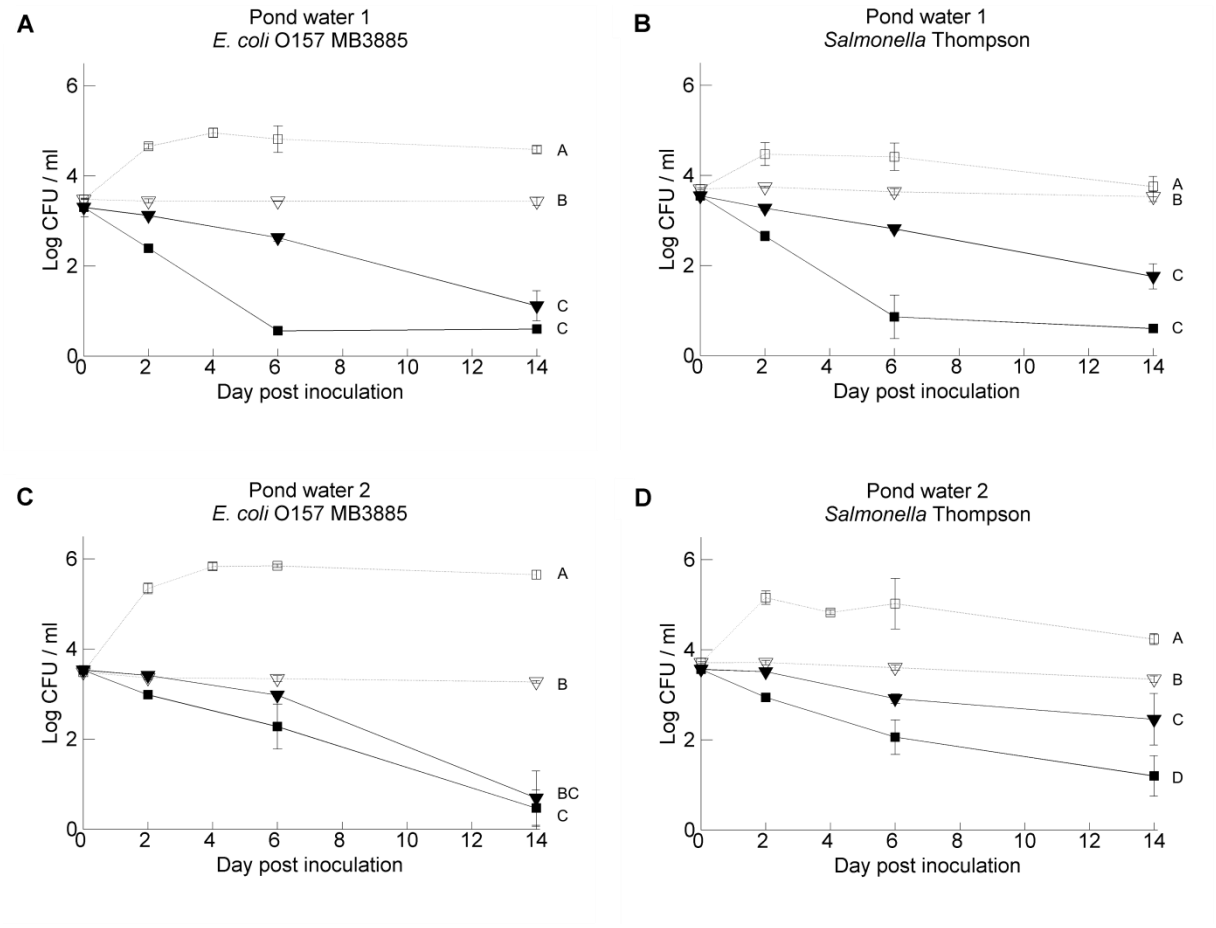


Figure 4.2: Survival of *E. coli* O157:H7 MB3885 (left) and *Salmonella* Thompson RM1987N (right) in 2 pond water samples with the following treatments: untreated water samples stored at 4°C (full line, ▼), filter sterilized water samples stored at 4°C (dashed line, ▽), untreated water samples stored at 20°C (full line, ■), filter sterilized water samples stored at 20°C (dashed line, □). The data show the mean of three analyzed vials and are calculated from the log transformed values of the pathogen population size. Error bars indicate standard deviations. Different letters indicate significant difference ($P < 0.05$) between means according to negative binomial regression.

4.3.2 Pathogen's survival in irrigation water; comparisons between water samples and different strains of each pathogen

The pathogen's survival was significantly different between the different irrigation water samples ($P < 0.001$). This was especially clear at 20°C, and less at 4°C. Besides that, irrigation water GW3 showed a very different pathogen survival profile. In general, similar survival trends were observed for *Salmonella* Thompson and *E. coli* O157:H7 MB3885 under the same experimental conditions, although some differences could be seen: *E. coli* O157:H7 MB3885 survived significantly better than *Salmonella* Thompson RM1987N in some test conditions, such as in sterilized GW1 ($P<0.001$, Figure 4.1 A-B) and sterilized PW2 at 20°C ($P<0.001$, Figure 4.2 C-D) and in untreated GW3 at 4°C ($0.001<P<0.01$, Figure 4.1 E-F). To test whether the observed differences were strain specific or species specific, the survival of two more strains, *Salmonella* Typhimurium MB4880 and *E. coli* O157:H7 NCTC12900, was tested in the untreated water samples stored at 20°C. The results are

shown in Figure 4.3. The survival of the two *Salmonella* strains was in general not significantly different from each other, whereas for the two *E. coli* O157:H7 strains a difference in pathogen level up to 2.0 log CFU/ml existed when residing in the PW2 water at day 14 after inoculation. In 4 of the 5 water samples *E. coli* O157:H7 NTCT12900 survived less well than *E. coli* O157:H7 MB3885, whereas the opposite was observed in GW3.

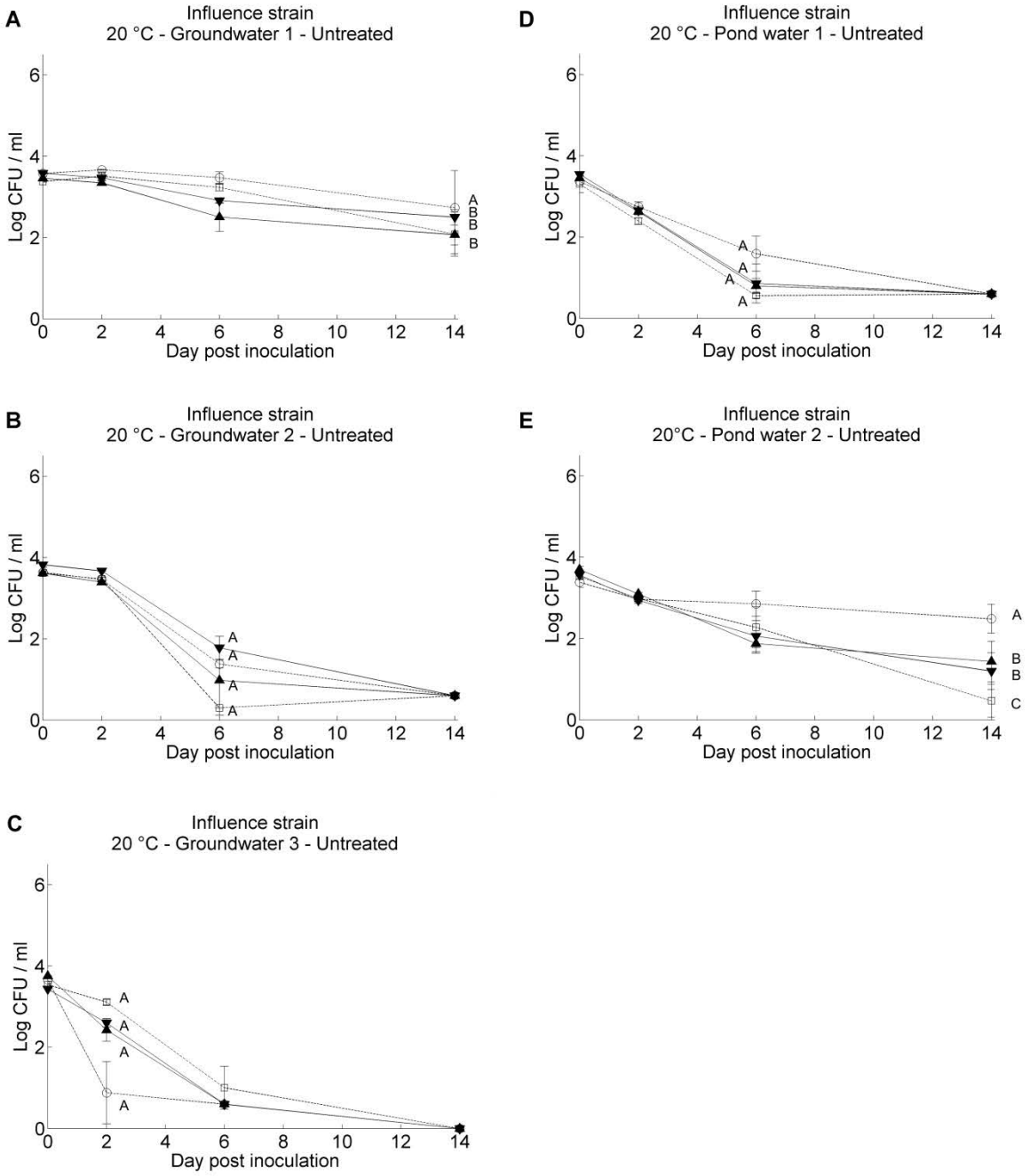


Figure 4.3: Survival of *Salmonella* Typhimurium MB4880 (▲), *Salmonella* Thompson RM1987N (▼), *E. coli* O157:H7 MB3885 (□) and *E. coli* O157:H7 NCTC12900 (○) in untreated irrigation water stored at 20°C. Pathogen suspensions in **(A)**, groundwater 1; **(B)**, groundwater 2; **(C)**, groundwater 3; **(D)** pond water 1 and **(E)** pond water 2. The data show the mean of three analyzed vials and are calculated from the log transformed values of the pathogen population size. Error bars indicate standard deviations. Different letters indicate significant difference ($P < 0.05$) between means according to negative binomial regression.

4.3.3 The resident background of heterotrophic microorganisms in the irrigation water samples

The population dynamics of the heterotrophic background microbiota are shown in Figure 4.4. The initial counts varied from 1.81 ± 0.30 log CFU/ml to 5.00 ± 0.08 log CFU/ml between the different irrigation water samples and increased during the 14-days storage experiment. Levels up to 6.98 ± 0.16 log CFU/ml were observed in GW2 at 4°C at day 14. The increase was faster but not higher at 20°C (within 2 days) than at 4°C. No statistically significant correlation could be found between the growth/die off rate of the pathogens and the heterotrophic background microbiota for any of the water samples. Furthermore, the counts of the heterotrophic bacteria in the inoculated water samples were not significantly different from the counts of the heterotrophic bacteria in the non-inoculated irrigation water samples.

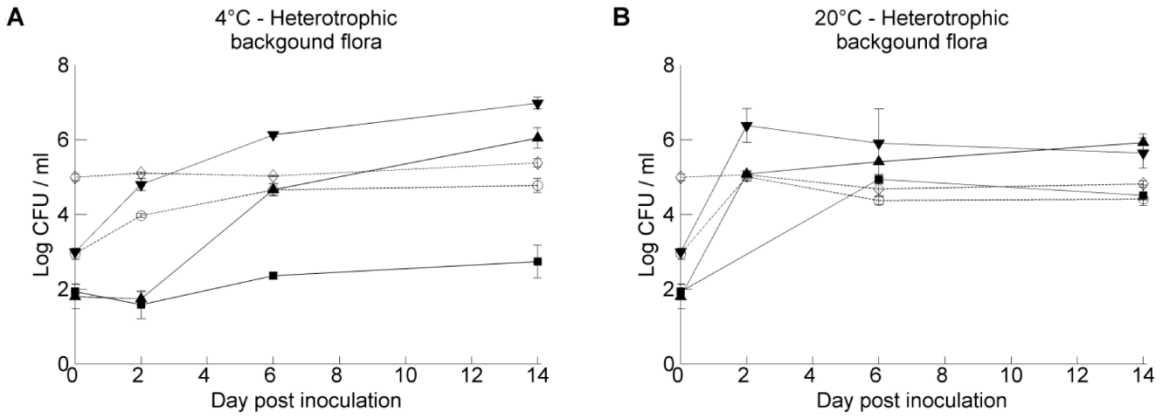


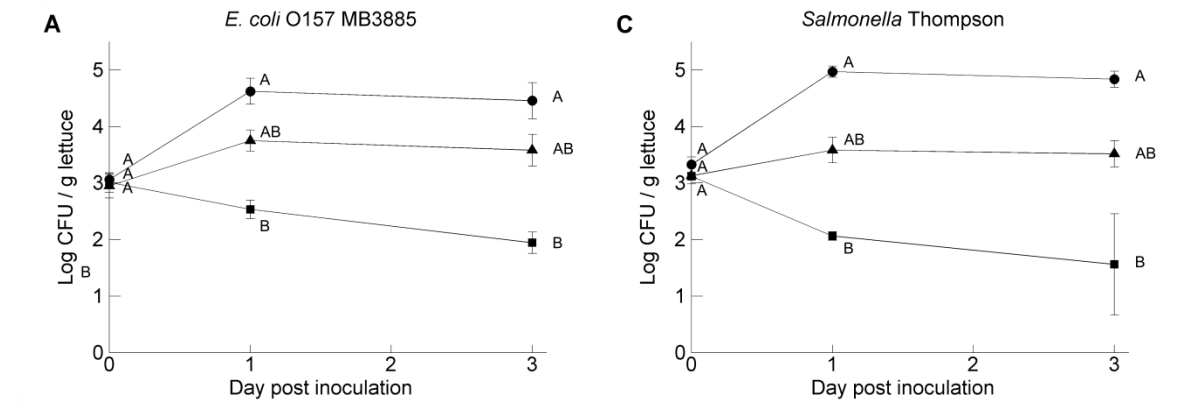
Figure 4.4: Plate counts of heterotrophic background microbiota residing in the five irrigation water samples (groundwater 1 (▲), groundwater 2 (▼), groundwater 3 (■), pond water 1 (◊), pond water 2 (○)) stored at 4°C **(A)** and 20°C **(B)**. The data show the mean of three analyzed vials and are calculated from the log transformed values of the heterotrophic background population size. Error bars indicate standard deviations.

4.3.4 Pathogen's survival on butterhead lettuce leaves; influence of the inoculum carrier

The results are shown in Figure 4.5. The preliminary test showed that the GFP plasmid remained present in both strains, green fluorescence was detected in more than 99% of the colonies up to the end of the test (day 10). When *Salmonella* and *E. coli* O157:H7 were suspended in PBS, GW3 or PW2 as carrier to inoculate lettuce leaves, significantly different concentrations of these pathogens were recovered from the leaves ($0.01<P<0.05$). The highest concentrations were recuperated from leaves inoculated with suspensions in PBS, and then followed by the variant in PW2. In contrast, when

inoculated in GW3 water, the amount of pathogens on the leaves declined. *E. coli* O157:H7 MB3885 and *Salmonella* Thompson behaved in a similar way.

Experiment 1:



Experiment 2:

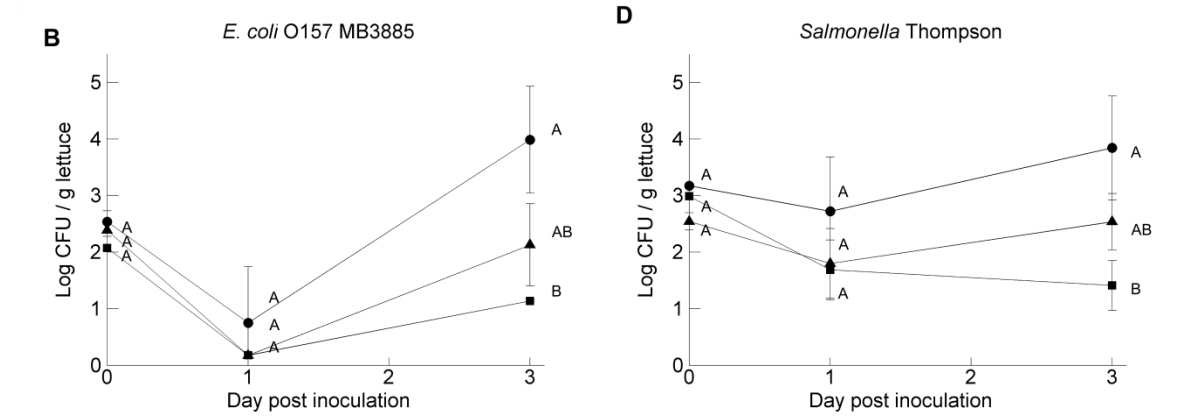


Figure 4.5: Levels of GFP labeled *E. coli* O157:H7 MB3885 **(A & B)** and GFP labeled *Salmonella* Thompson RM1987N **(C & D)** recovered from lettuce leaves at three time points after inoculation with the pathogens suspended in phosphate buffer-saline (●), pond water 2 (▲), or groundwater 3 (■). Data presented are from 2 independent experiments (experiment 1 :A & C; experiment 2 B & D) and are calculated from the log transformed values of the pathogen population size on 3 leaf samples and expressed per gram of leaf tissue. Error bars indicate standard deviations. At each day, different letters indicate significant difference ($P < 0.05$) between means of pathogen level according to Kruskall-Wallis non parametric test.

4.4 Discussion

This study was conducted to evaluate the extent to which enteric pathogens survive in irrigation water from different origin and quality and to have a first estimate of the implication for contamination of fresh green produce. We used butterhead lettuce as test plant. Lettuce cultivation in greenhouse is common in Belgium and irrigation water reservoirs were sampled on five different production sites. In the first part of our study, the survival of *E. coli* O157:H7 and *Salmonella* was followed in artificially inoculated irrigation water samples from different Belgian lettuce production sites. The influence of temperature, presence of resident aquatic biota, chemical water quality or

pathogen strain was tested. A schematic summary and tentative interpretation of our results performed at 20°C is shown in Table 4.3.

For natural waters, it is known that temperature has a significant effect on the survival of enteric pathogens (Steele and Odumeru 2004) with better survival at lower temperatures (Rice *et al.* 1992; Wang and Doyle 1998). This was also seen in the present experiment in the untreated water samples. But in the filter sterilized water samples, the opposite was observed. In three irrigation water samples, even growth of the pathogen could be noticed at 20°C. These results are in accordance with Vital *et al.* who have shown that growth of *E. coli* O157:H7 can be observed in sterilized natural freshwater at low carbon concentrations when the initial inoculation concentration is not higher than the so-called 'carrying capacity' of the water (Vital *et al.* 2008; Vital *et al.* 2010). This may also indicate that sterilized irrigation water could be a risk factor when the contamination event occurs after the sterilization treatment. By comparing the pathogen survival in both filter sterilized and untreated water samples, the influence of the presence of the heterotrophic background microbiota and the chemical water quality could be determined. In general, the survival of *E. coli* O157:H7 and *Salmonella* was significantly better in the sterilized water samples. This indicates that for these samples, competition with the resident aquatic microbiota may be responsible for the decline of the pathogens in the untreated water samples. Bacterial competition is a very complex process of which the current state of knowledge of the contributing factors (such as nutrient dynamics, concentrations of competing species) is very limited for natural waters (Vital *et al.* 2008). Furthermore, also the presence of protozoa may have influenced the survival of enteric pathogens in irrigation water. Ravva *et al.* has shown that selected types of protozoa preferentially engulf specific isolates of *E. coli* O157:H7 while some protozoa engulf the pathogen in the presence of specific nutrients (Ravva *et al.* 2010; Ravva 2013). This may be one of the factors that can explain the pronounced differences that were observed for the two *E. coli* O157:H7 strains, but the hypothesis could not be confirmed as protozoal counts were not performed.

Table 4.3: Schematic overview results of survival of *Salmonella* and *E. coli* O157:H7 in 5 irrigation water samples which were previously filter sterilized or not and subsequently stored at 20°C.

water sample	Initial level resident heterotro-phic bacteria	Endpoint pathogen level		Tentative interpretation		
		in presence of aquatic biota (in untreated water)	in absence of aquatic biota (in filter sterilized water)	combination of competitive survival and growth and nutrient availability	risk of pathogen transfer when using non-sterilized irrigation water	risk of pathogen transfer in case of pathogen contamination of sterilized water
PW1	high	low	very high	- important pathogen suppression by resident aquatic biota - nutrients for pathogen growth available	low	very high
PW2	medium	medium	very high	- weaker pathogen suppression by resident aquatic biota - nutrients for pathogen growth available	medium	very high
GW1	low	high	very high	-very low pathogen suppression (low bacterial background load) - nutrients for pathogen growth available	high	very high
GW2	medium	low	high	-important pathogen suppression by resident aquatic biota -limited nutrients for pathogen growth available -high Zn level	low	high
GW3	low	low	low	-important pathogen suppression but not by the resident aquatic biota the pathogen does not survive in this water, although the bacterial background does -high Zn level	low	low

In two water samples (GW2 and GW3) the pathogen behavior in the untreated samples could not (only) be explained by competition with the resident aquatic microbiota. In GW2 the survival of the pathogen was significantly better in the sterilized water sample but no growth could be observed, and in GW3 no difference in survival was seen between the untreated and filter sterilized water. This may indicate that for these water samples also the chemical water quality may have had a significant influence on the survival of the pathogen. One of the factors that may explain these results is the fact that water samples were characterized by a higher Zn concentration. For GW3 we could show that this high concentration originated most likely from galvanized irrigation pipes and irrigation storage tank as water which was sampled directly from the borehole reservoir was characterized by a much lower Zn concentration. When a survival experiment was conducted with this borehole water sample, growth of the pathogen could be observed in the sterilized water sample (Figure 4.6 & Table 4.4). Toxic effects of zinc on bacteria have been reported (Preston *et al.* 2000). It was shown that a concentration of 0.25 mg/l has a direct toxic effect (20 min) on *E. coli* and that a longer exposure time significantly increases the sensitivity of *E. coli* to metal pollutants (13). In a similar survival experiment as ours, Avery *et al.* (2008) found a significant negative correlation between the mean log CFU *E. coli* O157:H7 and log Zn concentration. In GW2, very high levels of zinc were found as well, yet differences in filtering did change the pathogen survival. This may indicate that the Zn-complex in the two water samples was not the same. Furthermore, other toxic chemical elements may have been present in GW3 which were not analyzed, or synergistic effects between different chemical elements could have occurred (Preston *et al.* 2000).

These results (influence competition microbiota, chemical composition) show high similarities with the survival of enteric pathogens in various other natural substrates such as manure and slurry (Semenov *et al.* 2007).

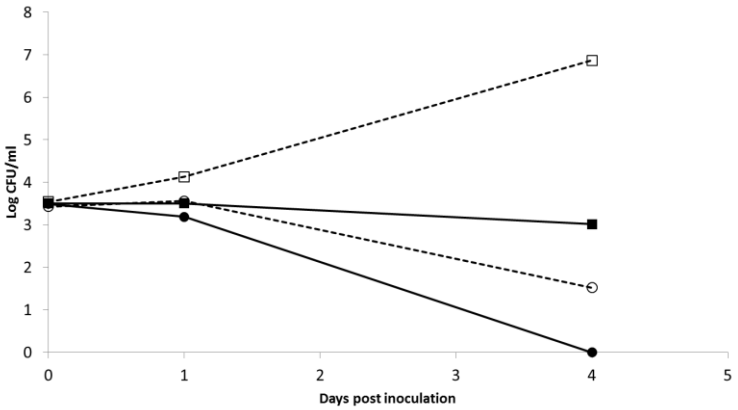


Figure 4.6: Survival of *E. coli* O157:H7 MB3885 in groundwater 3 sampled at the borehole reservoir and at the tap with following treatment: untreated borehole reservoir (full line, ■), filter sterilized borehole reservoir (dashed line, □), tap point untreated (full line, ●), filter sterilized tap point (dashed line, ○). Only one measurement was performed for each time point. Plate count data from TSA-counts.

Table 4.4: Metal concentrations measured in borehole reservoir and water taken from tap from groundwater 3 (independently sampled from the water sample used for the main experiments).

	Al	Ca	Fe	Mg	K	Na	Co	Cr	Cu	Mn	Ni	Zn
	µg/l	mg/l	µg/l	mg/l	mg/l	mg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l
borehole reservoir	143.9	99.0	360.0	14.9	8.94	20.1	BD	4.3	11.6	121.9	6.8	60.0
tap	BD	114.6	64.0	13.4	4.42	15.6	BD	0.1	2.8	0.4	0.4	2793.5

BD = below detection limit

Large differences could be observed between selective and non selective plate counts for some water samples, with lower count on selective medium. This indicates that the pathogens in these water samples were in a stressed condition and most likely sublethally damaged (Dinu *et al.* 2009).

In the second part of our study, two of the artificially inoculated water samples were used to introduce the pathogens onto butterhead lettuce leaves to evaluate their subsequent survival. These results were compared with PBS as inoculum carrier which is together with sterile distilled water and other standard sterile buffers commonly used. Only few studies have used irrigation water as inoculum carrier (Solomon *et al.* 2003; Barker-Reid *et al.* 2009), probably because this makes it more difficult to repeat the experiment in exactly the same conditions. To our knowledge, only Theofel and Harris (2009) and Choi *et al.* (2011) have investigated the influence of the inoculum carrier on the subsequent survival of the pathogen on leafy greens. Theofel and Harris did not find significant differences when Milli Q water, 0.1% peptone water, or pond water was used as an inoculum carrier to deliver *E. coli* O157:H7 to fresh-cut lettuce that was subsequently stored at either 5 or 20°C. They used an average starting inoculum level (± 3.5 log CFU/g lettuce) which was comparable with ours (3.1 ± 0.2 log CFU/g), but the inoculation method was different. They spotted 10 µl inoculum with 6.5 log CFU/ml in 3-5 drops on 10 g of lettuce whereas we chose to use the dip inoculation method. The inoculum density that we used in the present study was on average lower (log 3.5 ± 0.3 CFU/ml) but we used a 300 times higher inoculum volume (± 3 ml for 10 g of lettuce). With this inoculation method, the pathogen was more evenly distributed on the leaves. This may explain why we were able to observe a significant effect of the inoculum carrier, although also other experimental factors could have played a role (different strain, lettuce type, storage temperature, etc.). Choi *et al.* were able to see differences in *E. coli* O157:H7 survival on lettuce when sterile distilled water or peptone water as inoculum carrier was used, with better survival with peptone water. They used 100 µl inoculum for each leaf and suggested that the organic matter in peptone water protects *E. coli* O157:H7 from environmental stresses and/or provides nutrients to support colonization in an environment with 100% relative humidity. In our test, the pathogen's survival on the plant was comparably better when introduced in PBS, but with the current experimental design it was not possible to explain whether this effect was due to the absence of resident aquatic microbiota in the

sterile buffer, the chemical composition of the irrigation water samples or a combination of these factors.

We chose to perform the experiment with detached butterhead leaves with the same leaf age in conditions of high relative humidity in order to keep the variation as low as possible (Brandl and Amundson 2008; Theofel and Harris 2009). This is important when small differences in the survival of enteric pathogens on fresh produce need to be investigated. But even under these standardized conditions, the survival between the different repeats were significantly different although the same trends could always be observed: better survival on the lettuce when introduced with PBS as inoculum carrier, less survival when introduced with GW3. The tested high humidity conditions (100%) are, however, not typical of preharvest conditions. Under drier conditions, it is therefore likely that populations of both groups would have declined such as described previously and it would have been difficult to discern an effect of the irrigation water quality to the overall pathogen's survival (Brandl and Mandrell 2002; Van der Linden *et al.* 2013). In order to test the hypotheses that were put forward in this study and to investigate the impact of the irrigation water quality on enteric pathogen's survival on lettuce under commercial growth conditions in detail, further research is required. In such follow up studies a higher number of water samples should be investigated, and the experiments should be repeated in time, in order to take the chemical and microbiological variability of the water into account. Furthermore, it should be interesting to characterize the nutrient availability of the water samples in more detail e.g. by measuring the dissolved organic content (DOC) per unit microbial biomass (Franz *et al.* 2007a; Gu *et al.* 2012) and to determine the low pathogen levels more accurately e.g. by applying the MPN-method.

4.5 Conclusion

Our study confirms that the survival of *Salmonella* and *E. coli* O157:H7 may vary between different irrigation water samples. The individual pathogen's fitness for leaf colonization seems to be influenced by the quality of the irrigation water under conditions of high relative humidity.

Chapter 5:

Evaluation of an attachment assay on lettuce leaves with temperature and starvation stressed *E. coli* O157:H7

Redrafted from:

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M. & Heyndrickx, M. & (2014). Evaluation of an attachment assay on lettuce leaves with temperature and starvation stressed *E. coli* O157:H7 MB3885. Journal of Food Protection, 77(4): 549-557.

Preparation of the leaf discs used for the attachment assay

Abstract

Attachment of enteric pathogens such as *E. coli* O157:H7 to fresh produce is a crucial first step for contamination to occur and irrigation water is considered a potentially important preharvest introduction route. In a natural situation *E. coli* O157:H7 may be present in the irrigation water for some time and may, therefore, be starved. Most research, however, is performed with freshly cultured strains. The aim of this study is to examine the behavior of *E. coli* O157:H7 MB3885 under starvation stress in water used for overhead irrigation in the greenhouse and the consequence on its subsequent ability to attach to butterhead lettuce leaves. *E. coli* O157:H7 MB3885 was starvation stressed by introducing it at ± 7.5 log CFU/ml into phosphate buffered saline (PBS), sterile distilled water (SDW), or irrigation water (IW). The suspensions were stored at 4°C or 20°C, and used after 0, 2 and 6 days for an attachment assay on butterhead lettuce. *E. coli* O157:H7 MB3885 levels were determined by plating method and live/dead-qPCR technique. A decrease in plate-counts, an indicator of stress, was observed for most of the conditions, whereas a die-off, as revealed by the live/dead-qPCR-data, was only observed in IW stored at 20°C. Overall, stress appeared to be highest in IW and lowest in PBS. The stressed cells were still able to recover, even at 4°C, and to attach to the lettuce. Furthermore, our results show that standard laboratory solutions such as PBS and SDW may not be the best to simulate stressed cells in irrigation water in which the bacteria may behave significantly different.

5.1 Introduction

In recent years, different outbreaks with the pathogen *Escherichia coli* O157:H7 could be related to the consumption of fresh produce such as lettuce, baby spinach, fresh herbs etc. (Berger *et al.* 2010; Olaimat and Holley 2012). During primary production, fresh produce can become contaminated with pathogens by contact with feces or fecally contaminated run-off water, by contact or splashes from soil (when soils are fertilized with untreated manure), by untreated surface or wastewater used for irrigation, or pesticide application, or by unhygienic handling by workers during harvest (Olaimat and Holley 2012). Contaminated irrigation water is one of the main preharvest introduction routes of phytopathogenic bacteria for greenhouse grown produce (Cottyn *et al.* 2011), and has also been suspected as the cause of zoonotic outbreaks in the past (Nygård *et al.* 2008; Franz *et al.* 2009).

When water carrying *E. coli* O157:H7 is applied to greenhouse-grown leafy vegetables, a first step in the contamination process is attachment of the pathogenic bacteria to the leaves. Various studies showed that once the pathogens have attached to the leaves, they are very difficult to remove again. Normal washing procedures with water, but also using sanitation cannot guarantee safe fresh produce (Gomez-Lopez *et al.* 2008).

Efforts have been made to gain insight into the mechanisms that facilitate bacterial attachment to plant tissues (e.g. fruit, leafy vegetables, etc.). Mainly *Salmonella* and *E. coli* O157:H7 have been studied. Generally a two-step model is proposed to explain the attachment of bacterial cells to plant tissues. First, reversible attachment occurs and the bacterial cells can be removed by rinsing or a turbulent flow. This initial attachment is mediated by basic physical forces (Goulter *et al.* 2009). Then, irreversible attachment can take place. Studies demonstrated that this cannot be explained by a single mechanism but that different factors, such as the bacterial strains or species (Takeuchi *et al.* 2000; Barak *et al.* 2002; Ukuku and Fett 2002; 2006), bacterial cell properties (Ukuku and Fett 2002; 2006; Patel *et al.* 2011), produce surface (species, crop type, damage) (Seo and Frank 1999; Takeuchi *et al.* 2000; Takeuchi *et al.* 2001; Boyer *et al.* 2007; Patel and Sharma 2010; Kroupitski *et al.* 2011; Patel *et al.* 2011), and environmental conditions (Iturriaga *et al.* 2003; Hassan and Frank 2004), all may have an influence.

Previous attachment studies were performed with fresh inocula in suspensions of sterile distilled water or buffered solutions prepared in the laboratory (Barak *et al.* 2002; Ukuku and Fett 2002; Hassan and Frank 2004; Ukuku and Fett 2006; Boyer *et al.* 2007; Kroupitski *et al.* 2011; Patel *et al.* 2011). Under natural conditions, however, the pathogens may reside in irrigating water for an extended period of time before contamination and attachment to the leaves occurs. It has been shown that survival of enteric pathogens in water could lead to starvation stress (Chekabab *et al.* 2013). Starvation stress, in turn, leads to a rapid adjustment of the physiology of *E. coli* O157:H7 particularly by stress response induction (Allen *et al.* 2010; Jozefczuk *et al.* 2010). The general stress response regulator, sigma factor RpoS, triggers among others the expression of the filamentous type III secretion system, which is needed for the attachment of *E. coli* O157:H7 (Shaw *et al.* 2008; Dong and Schellhorn 2009). Little is known about the relationship between pathogen attachment and starvation (Haznedaroglu *et al.* 2008). To our knowledge, the effect of starvation on the attachment of *E. coli* O157:H7 to fresh produce has not yet been addressed.

The aim of this study is to obtain a better understanding of the starvation stress of *E. coli* O157:H7 MB3885 in water and its effect on leaf attachment. Attachment of *E. coli* O157:H7 MB3885 to butterhead lettuce discs was examined after exposure to irrigation water for 0, 2 or 6 days at 4 and 20°C, respectively, and compared to the commonly used laboratory suspensions of *E. coli* O157:H7 MB3885 in sterile distilled water (SDW) and sterile phosphate buffered (PBS) saline.

5.2 Material and methods

5.2.1 Bacterial strain and growth conditions

E. coli O157:H7 MB3885, isolated from beef carpaccio and kindly donated by the Scientific Institute for Public Health (Brussels, Belgium) carries the *eae*-gene and lacks Shiga toxin genes (*stx1* and *stx2*) and was used as non-toxigenic surrogate strain for the Shiga toxin producing (STEC) serotype O157:H7 for biosafety reasons. The absence of the *stx*1 and *stx*2 genes and the presence of other virulence genes *eaeA* (intimin), *ehx* (enterohemolysin), *espP* (extracellular serine protease) and *katP*

(catalase-peroxidase) were confirmed by conventional PCR by Verstraete *et al.* (2013). For each experiment, the strain was streaked from a glycerol frozen stock maintained at −70°C onto a tryptone soy agar plate (TSA; Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h. One colony from the plate was transferred to 10 ml of tryptone soy broth (TSB; Oxoid, Basingstoke, UK) and incubated at 37°C for 18 h at 200 rpm. Bacteria were washed twice with sterile deionized water (SDW) by centrifugation at 13000×g for 1 min.

5.2.2 Suspension preparation

The *E. coli* O157:H7 MB3885 cells were introduced into three types of aqueous solutions. Sterile phosphate buffered saline (PBS, pH 7.4) was chosen as it is isotonic to the interior of the bacterial cell and sterile distilled water (SDW) was chosen to represent an environment that is hypotonic to the bacterial cell. Both types of suspension media are frequently used in attachment studies (Barak *et al.* 2002; Ukuku and Fett 2002; Hassan and Frank 2004; Ukuku and Fett 2006; Boyer *et al.* 2007; Kroupitski *et al.* 2011; Patel *et al.* 2011) to simulate irrigation water. Finally, irrigation water (IW) (not sterile) from the experimental greenhouse of ILVO was collected twice in sterile, pre-rinsed, 1 l bottles. A chemical analysis of the irrigation water was performed (Table 5.1). One night before inoculation 125 ml of IW, sterile PBS and SDW were put in the dark at 4°C and at 20°C. The *E. coli* O157:H7 pellet was resuspended in SDW, PBS or IW stored at 4°C or 20°C to a final concentration of ± 7.5 log colony-forming units (CFU) per ml. For each of the two independent repetitions of the experiment, 3 aliquots of 37 ml were prepared in 50 ml tubes for each condition, i.e. each combination of suspension type and temperature (SDW-4°C, SDW-20°C, PBS-4°C, PBS-20°C, IW-4°C, IW-20°C) (Figure 5.1, a). These were stored at 4°C or at 20°C in the dark during the whole experiment. Two hours after inoculation (day 0), and 2 and 6 days after inoculation, one tube of each condition was divided into 3 aliquots of 12 ml into three 50 ml tubes which were used for the attachment assay (Figure 5.1, f) and 2 times 500 µL in two sterile light-transparent 600 µL microcentrifuge tubes for the PMA-qPCR (Figure 5.1, b).

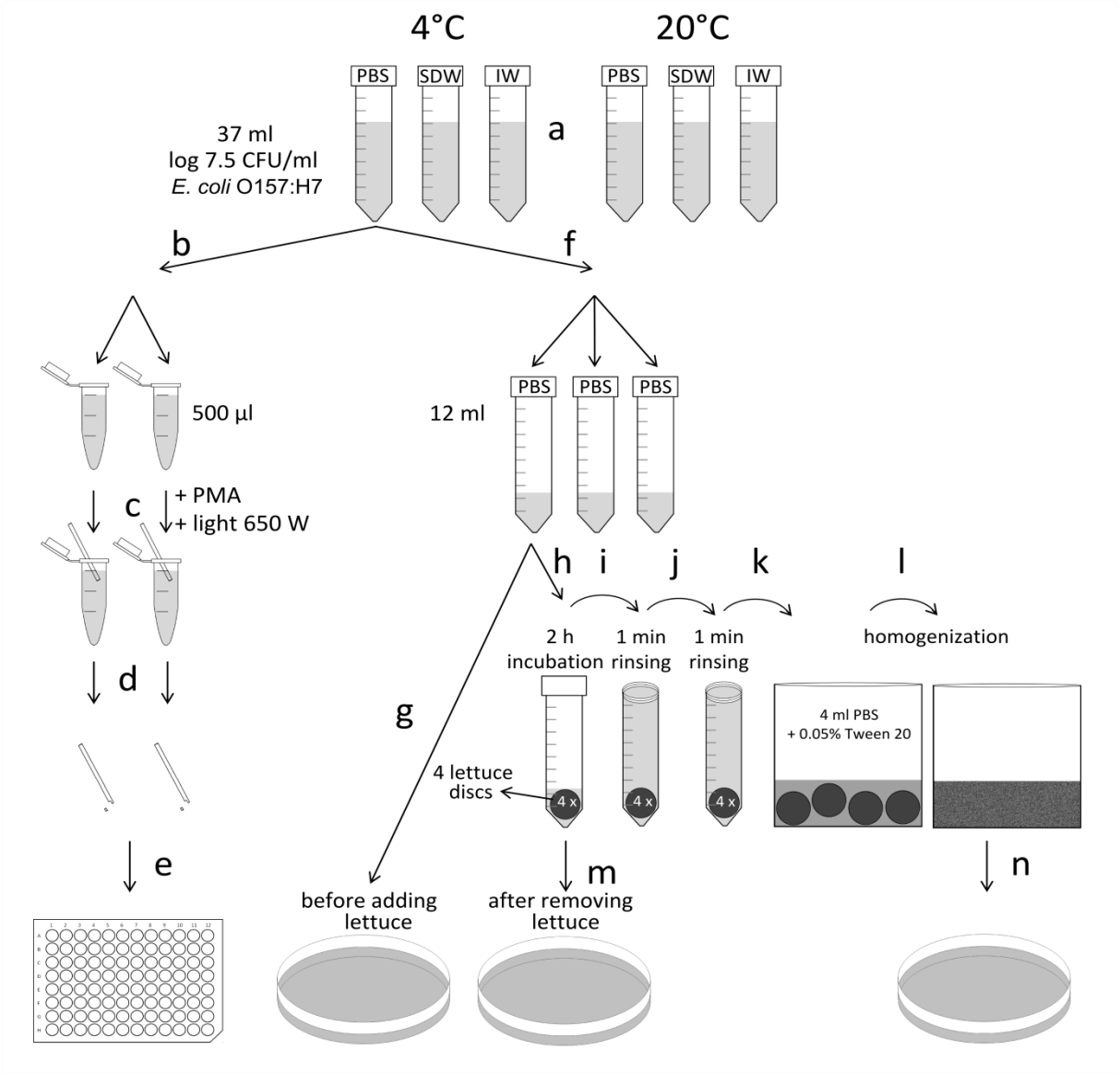


Figure 5.1: Schematic overview of the attachment assay performed on day 0, this assay was repeated at day 2 and 6. Aliquots of 37 ml sterile phosphate buffered saline (PBS), sterile distilled water (SDW) or irrigation water (IW) inoculated with log 7.5 CFU/ml *E. coli* O157:H7 were made an stored at 4°C and 20°C (a). Two times 500 µL were removed for the PMA-qPCR (b). One vial was treated with PMA and an FTA-strip was dipped for 1 s in the untreated or PMA-treated sample and placed in a sterile 1.5 ml microcentrifuge tube (c). A piece of 1 x 1 mm was cut with a scalpel from each FTA-strip (d). Each piece was placed in a 0.6 ml microcentrifuge tube and the DNA-extraction was performed as described by the manufacturer. These pieces were used for the qPCR (e). The other 36 ml was divided into 3 aliquots of 12 ml which were used for the attachment assay (f). Before the attachment assay was performed, the number of culturable *E. coli* O157:H7 MB3885 in the suspensions was determined by selective plating (g). Then, four lettuce discs were submerged in 12 ml of each suspension and stored for 2 h at 4°C or 20°C respectively (h). Lettuce pieces were removed and the number of CFU/ml of *E. coli* O157:H7 MB3885 in the suspensions was determined again by selective plating (m). The lettuce pieces were rinsed twice for 1 min each in 50 ml SDW to remove unattached bacteria (i,j). The discs were transferred into 4 ml PBS + 0.05% Tween 20 in sterile filter bags (k) and ground (l). The number of bacteria attached to the lettuce was determined by selective plating (n).

Table 5.1: Chemical water characteristics of the irrigation water samples used for the two repeat experiments

	pH-H_2O	EC	Fe	Cu	Zn	Mn	Mg	Ca	K	Na	NO_3^-	NO_2	NH_4^-	SO_4	Cl
	-	µS/cm	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
Sample 1	7.47	718.0	0.03	< 0.01	0.18	< 0.01	14.92	110.74	8.56	12.77	1.4	< 0.3	< 0.3	130.3	41.2
Sample 2	7.24	675.0	0.04	< 0.01	0.52	0.01	15.08	102.06	8.63	12.57	1.0	< 0.3	< 0.3	133.3	42.7

EC: electrical conductivity

5.2.3 *E. coli* O157:H7 MB3885 culturability in the suspensions determined by plating technique

The survival of the pathogen in the suspensions was determined at 0, 2 and 6 days post inoculation. Therefore, a 100 µl sample (Figure 5.1, g, 3 tubes per condition) was removed from the tubes before the attachment assay was performed, serially diluted in 0.1% peptone water and plated on cefixime-tellurite sorbitol MacConkey agar with a top layer of TSA (CT-SMAC-TAL) to determine the number of colony forming units (CFU) per ml (Figure 5.1, g). This selective plating technique offers the possibility for recovery of injured cells as described by Hajmeer *et al.* (2001). For one of the 2 repetitions, the number of CFU/ml of *E. coli* O157:H7 MB3885 in the suspensions was also determined after the attachment assay was performed and the lettuce discs (see section 'attachment assay') were removed from the suspensions (Figure 5.1, m).

5.2.4 Plant growth conditions

Pelletized butterhead lettuce seed (*Lactuca sativa* L. var. *capitata* 'Alexandria RZ') was obtained from Rijk Zwaan Distribution B.V., De Lier, The Netherlands. The plants were grown in pots of 20 cm diameter in the experimental greenhouse of ILVO to fully headed, mature plants (16 weeks of age).

5.2.5 Lettuce leaf discs preparation

Lettuce leaf discs were prepared as described by Kroupitski *et al.* (Kroupitski *et al.* 2011) with some adaptations. The outermost leaves of the lettuce crops were removed and the next 2 layers of leaves (± leaves 15-20) were detached and used for the experiments. Leaf discs (2.3 cm in diameter) were cut using the rounding of an aseptic 50 ml polypropylene tube as a template. Kroupitski *et al.* (2011) have described for *Salmonella* that the attachment to different leaf regions is highly variable, therefore only discs were prepared from the central region of the leaf. From each leaf, one disc left and one disc right from the vein was removed (see picture front page Chapter 5).

5.2.6 Attachment assay

The attachment assay was performed as described by Kroupitski *et al.* (2011) with some minor modifications. At day 0, day 2 and day 6, 4 lettuce discs, derived from 4 different leaves from different plants were submerged in 12 ml of each inoculum for 2 h at 4°C or 20°C (Figure 5.1, h). Lettuce pieces were then rinsed twice for 1 min each in 50 ml SDW to remove unattached bacteria (Figure 5.1, i,j). The discs were transferred into 4 ml PBS + 0.05% Tween 20 in sterile filter bags (Figure 5.1, k) and ground (Homex 6, Bioreba) until a homogenous mixture was obtained (Figure 5.1, l). The bacterial suspension was serially diluted in 0.1% peptone water and the number of CFU was determined by plating on CT-SMAC-TAL (Figure 5.1, n).

5.2.7 PMA-treatment

From the two 500 µl subsamples which were removed from the suspensions prior to the attachment assay (Figure 5.1, b), one was treated with propidium monoazide PMA (Biotium, Hayward, CA) as described by Yanez *et al.* (2011) with some modifications (Figure 5.1, c). Briefly, PMA was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) to obtain a stock concentration of 20 mM and stored at -20°C in the dark. A total of 1.25 µl of PMA solution was added to 500 µl of sample (final PMA concentration of 50 µM). After 5 min incubation in the dark (with occasional mixing), samples were exposed to light for 5 min using a 650W halogen light source. The sample tubes were placed horizontally on ice (to avoid excessive heating during light exposure and to maximize light exposure) in a distance of approximately 20 cm from the light source. A PMA-treatment could not be performed on the homogenized lettuce leaf solution as this solution was not transparent enough for the light exposure step in the protocol.

5.2.8 DNA isolation

An FTA-card (Whatman FTA Technology, Whatman International Ltd, United Kingdom) was cut into strips of 2 x 30 mm. An FTA-strip was dipped for 1 s in the untreated or PMA-treated sample and placed in a sterile 1.5 ml microcentrifuge tube to allow to dry for at least 2 h in a biosafety hood. The microcentrifuge tubes with FTA-strips could then be stored in the dark at room temperature. A piece of 1 x 1 mm was cut with a sterile scalpel from each FTA-strip (Figure 5.1, d). Each piece was placed in a 0.5 ml microcentrifuge tube and the DNA-extraction was performed as described by the manufacturer (Whatman FTA Technology, Whatman International Ltd, United Kingdom). Shortly, the samples were washed three times with 100 µl FTA-purification reagent, two times with Tris-EDTA buffer and dried for at least 1 h in the biosafety hood. These pieces were used in the qPCR (Figure 5.1, e).

5.2.9 Quantitative PCR with untreated and PMA-treated samples (PMA-qPCR)

The quantification of the strain was based on the detection of the *eae*-gene. Verstraete (2012) designed the *eae* primers that were used and are listed in Table 5.2. The qPCR assay was carried out in a 20 µl volume containing 1 × TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), primers and probe with a final concentration of 300 nM of each primer and 100 nM probe and a washed FTA-piece. qPCR was performed on a LightCycler® 480 (Roche Diagnostics) using the LightCycler® 480 software, with the following amplification program: incubation at 50°C for 2 min to eliminate carry-over contamination followed by an initial activation step of the enzyme at 95°C for 5 min and 40 cycles of 95°C for 15 s and 1 min annealing and elongation at 60°C. White LightCycler® 480 Multiwell Plate 96 (Roche Diagnostics) were used with the matching LightCycler® 480 Sealing Foil (Roche Diagnostics).

Table 5.2: Primers and probes used for qPCR quantification of *E. coli* O157:H7 MB3885. Corresponding nucleotide positions in sequences of indicated EMBL/Genbank accession numbers are given.

Primer/ probe	Sequence (5'- 3')	Position (5'- 3')	Accession number
Eae-F	GGA AGC CAA AGC GCA CAA	1507-1524	AF025311
Eae-R	GGC ICG AGC IGT CAC TTT ATA A	1593-1572	AF025311
Eae-P[a]	TAC CAG GCT ATT TTG CCI GCT TAT GTG C	1528-1555	AF025311

[a]Probe tagged with black hole quencher (BHQ-1) and a FAM fluorescent label (Eurogentec)

5.2.10 Control experiments

As a control it was determined whether the inoculum density influences the attachment ratio of the pathogens. Therefore, serial dilutions of freshly grown *E. coli* O157:H7 MB3885 suspended in sterile distilled water were made from log 8 to log 4 CFU/ml. For each dilution, 3 aliquots of 12 ml were made in 50 ml sterile polypropylene tubes. These were directly used to perform the attachment assay as described above. Negative control experiments were performed with non-inoculated lettuce pieces. For these samples, no bacterial growth was observed on the selective medium (CT-SMAC-TAL).

5.2.11 Statistical analyses

Each attachment assay and the control experiments were performed in triplicate. Two independent repetitions were performed on different days with different lettuce and suspensions. The determination of the pathogen level in the suspensions when the attachment assay was performed and the lettuce discs were removed (Figure 5.1, m) was performed for one representative repetition (in triplicate). Data were subjected to statistical analysis software SPSS (IBM SPSS Statistics 19. A generalized Poisson regression was used for the analysis of the attachment assay, one-way analysis

of variance (ANOVA) for the PMA-qPCR results and the resuscitation experiment with Least Significant Difference (LSD) test as post-hoc test for the PMA-qPCR.

5.3 Results

The survival of *E. coli* O157:H7 MB3885 was monitored for 6 days in three different suspensions: sterile phosphate buffered saline (PBS), sterile distilled water (SDW) and a groundwater irrigation water sample (IW) (Figure 5.2, A-C). The suspensions were stored at 4°C or 20°C for up to 6 days and were used at days 0, 2 and 6 for an attachment assay on butterhead lettuce discs (Figure 5.2, D-F).

5.3.1 *E. coli* O157:H7 MB3885 culturability and survival in the suspensions

The culturability of *E. coli* O157:H7 was monitored by a selective plating technique on the suspensions before the attachment assay was performed (Figure 5.1, g). The results are presented in Figure 5.2 (A-C). For *E. coli* O157:H7 MB3885 suspended in PBS, no marked decrease in culturability could be observed at both 4°C and 20°C after 6 days, whereas this could be seen in SDW and IW. In SDW, similar average declines were observed at 4°C (0.64 log CFU/ml, $P<0.001$) and at 20°C (0.44 log CFU/ml; $P<0.05$), whereas for IW an almost 150 times larger decline was observed at 20°C (2.91 log CFU/ml, $P<0.001$) compared with 4°C (0.74 log CFU/ml; $P<0.001$).

A life/dead qPCR with untreated and PMA-treated samples was performed to determine whether the observed decreases in plate counts were caused by cell death and/or by inculturability of the pathogen (Figure 5.3). qPCR-data only revealed a die-off of *E. coli* O157:H7 in IW stored at 20°C at day 6. For all other conditions, no significant differences in copy number between the untreated and PMA-treated samples could be seen.

5.3.2 Absolute *E. coli* O157:H7 MB3885 attachment to the lettuce discs

The attachment of *E. coli* O157:H7 to lettuce discs was determined by selective plating (Figure 5.1, n). The results are shown in Figure 5.2 (D-F). When freshly prepared suspensions (day 0) were used, no significant differences in attachment ratio of *E. coli* O157:H7 MB3885 could be seen between the different suspensions, except for *E. coli* O157:H7 MB3885 suspended in PBS at 4°C which showed on average a very slight but significantly lower (0.27 log CFU/g) attachment ratio compared with the other conditions. No significant difference in the absolute number of attached *E. coli* O157:H7 MB3885 to the lettuce discs could be found when PBS and SDW-suspensions that were stored for 2 and 6 days at 4°C and 20°C were compared with their the respective suspensions prepared at day 0. For *E. coli* O157:H7 MB3885 suspended in IW, however, a significant decrease in total number of attached cells was seen at both 4°C and 20°C for the suspensions stored for 6 days in comparison with the freshly cultured cells at day 0 ($0.01<P<0.05$ and $P<0.001$ respectively).

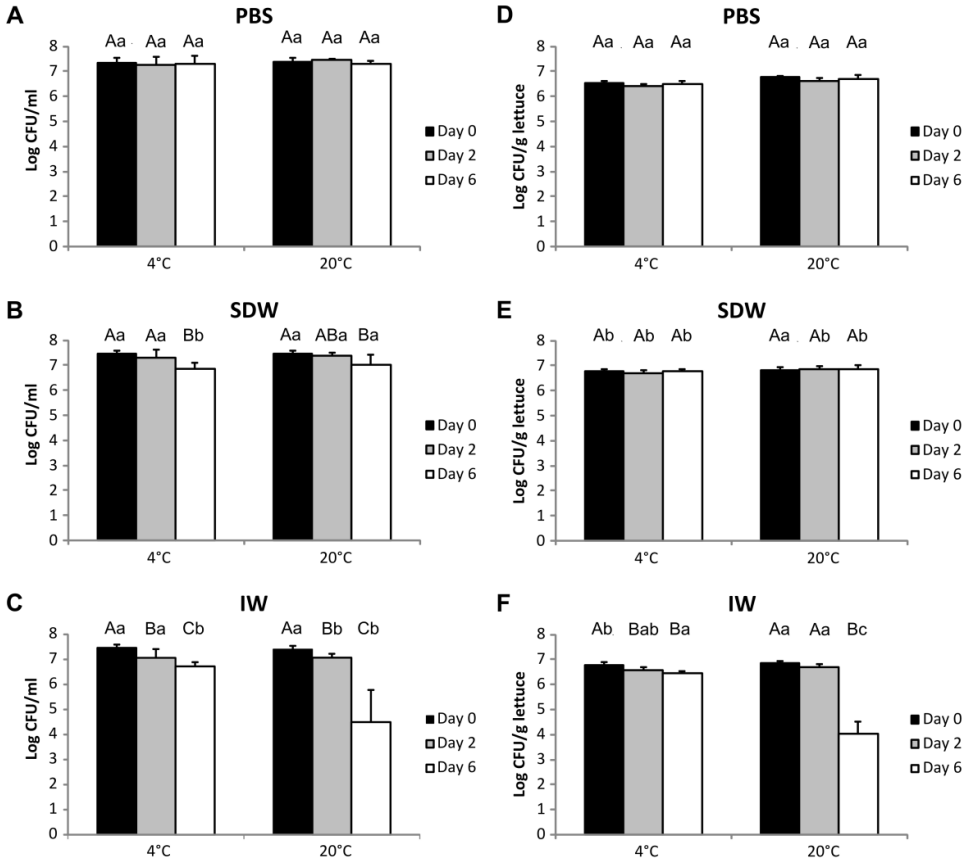


Figure 5.2: A-C Survival of *E. coli* O157:H7 MB3885 in the different suspensions stored at 4°C or 20°C; D-F Total number of attached *E. coli* O157:H7 MB3885 to lettuce leaf discs when the attachment assay was performed with *E. coli* O157:H7 MB3885 present for 0, 2 and 6 days in the different suspensions. The data represent the average log CFU/ml suspension or log CFU/g lettuce and the standard deviation of two independent experiments performed in triplicate. Different letters indicate significant difference according to generalized Poisson regression ($P < 0.05$) in the *E. coli* O157:H7 MB3885 level between the different days in the suspensions at 20°C or 4°C (capital letters, horizontal comparison per temperature), or in the *E. coli* O157:H7 MB3885 level on a certain day, between the different suspensions (lowercase letters, vertical comparison).

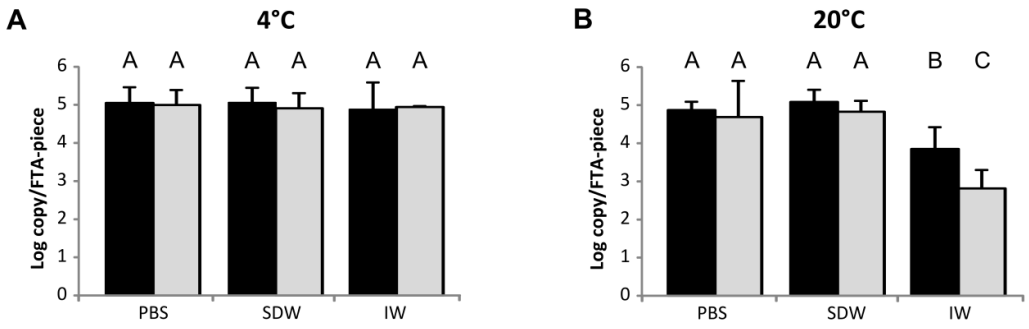


Figure 5.3: PMA-qPCR results based on the detection of the *eae*-gene for untreated (black bars, DNA from dead and alive cells detected) and PMA-treated (grey bars, DNA from alive cells detected) *E. coli* O157:H7 MB3885 samples from the different 6-days old suspensions stored at 4°C or 20°C. The data represent the average log copy/FTA-piece and standard deviation of two independent experiments performed in triplicate. Different letters indicate significant difference ($P < 0.05$) between means according to ANOVA with LSD as Post-Hoc Test.

5.3.3 Comparison of the attachment ratio of stressed and freshly cultured *E. coli* O157:H7 MB3885

To be able to compare the attachment ratio of the stressed *E. coli* O157:H7 MB3885 cells with unstressed cells, suspensions with the same pathogen concentration have to be compared as the attachment ratio of the bacteria may be inoculum density dependent. Therefore, the relationship between inoculum level of a SDW suspension with freshly cultured *E. coli* O157:H7 MB3885 and its attachment ratio to lettuce was determined (reference line, Figure 5.4). A log-log linear relationship with a high correlation (R^2 = 0.9986) could be seen. The data of the suspensions stored for 6 days were then plotted against this reference line to be able to compare the relative attachment of stressed and freshly cultured cells (grey symbols, Figure 5.4). It can be seen that some data points (SDW 4°C, IW 4°C) are situated left from the reference line. This indicates that for these samples, the pathogens seemed to attach slightly better compared with the freshly cultured cells, whereas *E. coli* O157:H7 MB3885 attachment in IW at 20°C seemed to be less than expected.

Furthermore, the pathogen level in the suspensions was also determined after the attachment assay was performed (Figure 5.1, m). Differences could be seen at day 2 (significant increase for SDW stored at 4°C at day 2 (0.01< P<0.05), and a decrease for PBS stored at 20°C (0.01< P<0.05)). With suspensions stored for 6 days, a significant increase in plate counts could be observed for all the suspensions stored at 4°C (0.01< P<0.05) with a maximum observed difference of 0.64 log for SDW, while a significant decrease was seen for irrigation water suspensions stored at 20°C (P<0.001). No significant differences were observed at day 0. When these plate counts are plotted against the reference line, the data fit much better to this line (day 6, white symbols, fig 4, for day 0 and day 2, see supplemental information from
http://studwww.ugent.be/~ivdlinde/Supplemental_information.html

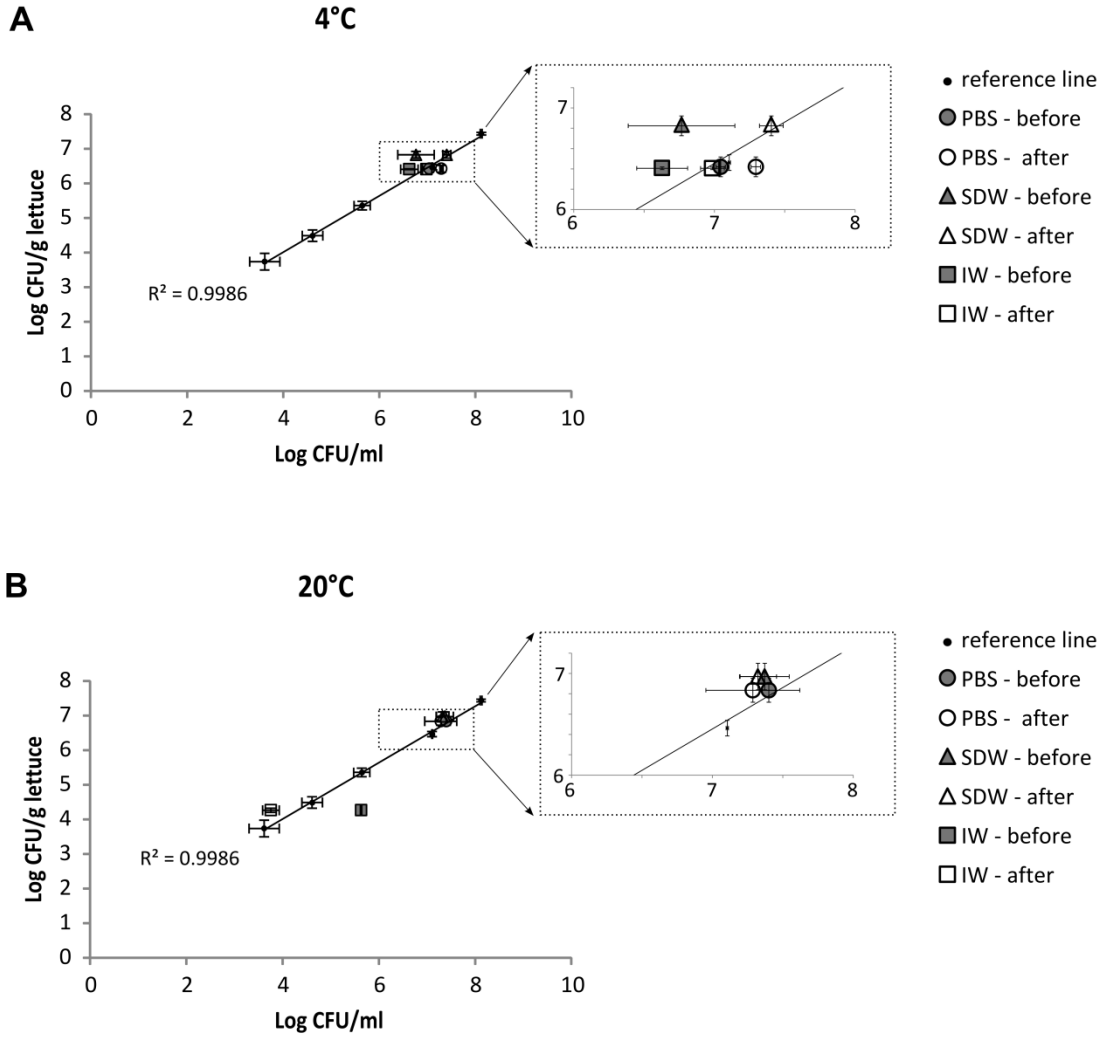


Figure 5.4: Comparison of the attachment of *E. coli* O157:H7 MB3885 present in the different suspensions for 6 days with freshly cultured cells. The black line shows the relationship for freshly cultured cells between the pathogen level in a SDW-suspension and the number of pathogens that were able to attach to lettuce discs. The data of the pathogen counts in 6-day old suspensions were plotted against this reference line. The pathogen level in the suspensions was determined before (grey symbols, see also Figure 5.1, g) and after (white symbols, see also Figure 5.1, m) the attachment assay was performed. PBS (○), SDW (△) and IW (□). Results from one of the two independent repeats shown in Figure 5.2 and 5.3. are shown for 6 day old suspensions stored at 4°C (A) and 20°C (B)

5.4 Discussion

The aim of the present study was to investigate whether the efficiency of leaf attachment differs between starvation stressed *E. coli* O157:H7 MB3885 cells and freshly cultured cells. Starvation was induced by suspending the pathogens in nutrient poor or nutrient free solutions for up to 6 days.

For *E. coli* O157:H7 MB3885 suspended in PBS, nor the culturability, or survival changed significantly during 6 days exposure to PBS at both 4°C and 20°C. With the methods used, indication of stress (defined as the change in culturability of the cells) could not consistently be observed in the suspensions, although studies using molecular methods have indicated changes in the outer membrane proteins (OMP) when *E. coli* O157:H7 after growth in M9, was starved for 6 days in PBS (Muela *et al.* 1999).

Attachment to the lettuce leaves was slightly lower for *E. coli* O157:H7 MB3885 cells exposed to PBS at 4°C compared to the other used conditions. Reduced attachment at lower temperatures was already described for gram negative bacteria (Fletcher 1977; Takeuchi *et al.* 2001), however, the process seems to be influenced by the type of suspension medium, as this effect could not be observed with SDW or IW-suspensions.

For *E. coli* O157:H7 MB3885 suspended in SDW or IW at 4°C, a decline in plate counts was observed, whereas no significant differences in gene copy number between the corresponding untreated and PMA-treated samples could be seen with qPCR. This may indicate that the observed decline by plate counts was not caused by cell death, but by inculturability of the cells on the CT-SMAC-TAL plates. A gradual loss in culturability was shown to be the first manifestation of cellular stress in the aquatic systems (Muela *et al.* 1999) and may be the precursor of the so called viable but non culturable state (VBNC). There are different definitions of this state, but in general it is accepted that bacteria in the VBNC state fail to grow on the routine bacteriological media on which they would normally form colonies, although they are still alive (Oliver 2000). Despite their typical low levels of metabolic activity, VBNC-cells are again culturable upon recovery. Most investigators consider the VBNC state to be a response to environmental stress that allows the cell's survival (Oliver 2010). It is known that especially at low temperatures, bacteria may go into the VBNC state (Rigsbee *et al.* 1997). Also Mizunoe *et al.* (1999) showed that *E. coli* O157:H7 starts to lose culturability after 5 to 8 days in SDW stored at 4°C.

For the attachment assay at 4 °C, a small increase in culturability of the pathogen after 6-days at 4 °C ($0.01 < P < 0.05$) was seen when lettuce leaf discs had been added to the suspensions two hours before the attachment assay, this was especially clear in SDW. The observed increase cannot be explained by cell growth as *E. coli* is not able to grow at 4°C, so probably this is recovery due to leakage of nutrients or other chemicals such as H_2O_2 from the leaf discs in the suspension. Kyle *et al.* (2010) have shown that *E. coli* O157:H7 exhibits enhanced survival in response to H_2O_2 challenge after exposure to Romaine lettuce lysates for 30 minutes. Enhanced resistance against H_2O_2 may be related to recovery from the VBNC-state (Oliver 2005).

It has been reported for *Listeria monocytogenes* and *E. coli* O157:H7 that the VBNC-status can be induced on leafy greens (Dreux *et al.* 2007a; Dinu and Bach 2011). Our results indicate that stressed *E. coli* O157:H7 MB3885 cells are able to attach to lettuce. Moreover, if the recovery effect is taken into account, no differences in attachment can be observed between stressed and freshly cultured cells.

For the attachment assay at 20°C, no significant die-off could be observed in SDW and PBS, whereas in IW a die-off and a loss of culturability was observed ($P < 0.001$). These results were clearly different from the results observed at 4°C. It is known that in environmental water samples, the microbial background may compete with *E. coli* O157:H7 for nutrients, but also predators or inhibitors such as toxic chemicals (e.g. heavy metals) may be present (Wang and Doyle 1998; Cho and Kim 1999; Artz and Killham 2002; Avery *et al.* 2008). Bacterial competition is higher at ambient temperature than at 4°C. The plating technique, however, did not allow us to investigate the effect of background microbiota in the inoculated IW sample because the *E. coli* O157:H7 inoculum level (± 7.5 log CFU/ml) was much higher than the natural background microbiota present (± 3 log CFU/ml, data not shown). Predators such as flagellates were not observed but the water sample was characterized by a high Zn-concentration, which can be toxic for bacteria when they are metabolically active (Bucheder and Broda 2005; Avery *et al.* 2008). In general, our results show that the behavior of *E. coli* O157:H7 MB3885 can be totally different in irrigation water compared to SDW or PBS.

In our study we investigated the influence of starvation (induced by suspension in nutrient poor or free solutions) and temperature on the attachment ratio of *E. coli* O157:H7 MB3885 to lettuce leaf discs. We have shown that for this strain the attachment ratio for the stressed, but still alive, cells is about the same as for the unstressed, freshly grown cells. Strain variation with respect to attachment to surfaces is well established for serotype O157:H7 (Rivas *et al.* 2007; Patel *et al.* 2011). It is, therefore, useful for future research to investigate the possible strain variation in the attachment of stressed cells. The results also indicate that standard laboratory solutions such as PBS and SDW may not be the best to simulate stressed cells in irrigation water, in which the bacteria may behave significantly different. Apart from the live/dead qPCR, other techniques such as microscopy coupled with nuclear stains used in live/dead staining assays, e.g. acridine orange could be used as extra confirmation. Further investigations are required as well in order to optimize the attachment protocol with stressed cells. The

Chapter 6:

Gene expression in *E. coli* O157:H7 Sakai present as epiphyte during cultivation of butterhead lettuce

Van der Linden I., Cottyn B., Uyttendaele M., Vlaemynck G., Heyndrickx M., Maes M., Holden N.

Experimental set-up of the lettuce plants (left) and lettuce washing to remove the pathogens (right)

Abstract

Numerous outbreaks with enteric pathogens such as *Escherichia coli* O157:H7 have been linked to consumption of fresh leafy vegetables. While the environmental factors which may influence the survival and proliferation of *E. coli* O157:H7 on growing plants are already intensively studied, little is known about the underlying genetic mechanisms. Whole genome transcriptional profiles were generated from *E. coli* O157:H7 cells (isolate Sakai, *stx*-) inoculated on the leaves of growing butterhead lettuce. Four-week old plants were inoculated through spray inoculation and placed in a growth chamber at 18°C and 75% relative humidity. One hour and 2 days after inoculation, RNA was extracted from the *E. coli* O157:H7 cells recuperated from the leaves, and as a control, from the suspension used as inoculum. The reverse-transcribed DNA was then analyzed on *E. coli* microarray slides (Agilent). A total of 273 *E. coli* O157:H7 Sakai genes (5.04% of the whole genome) was significantly up or down regulated by at least twofold ($P<0.01$) when the pathogen was associated with the lettuce leaves. Almost 40% of these genes were poorly characterized or had an unknown function. One hour after inoculation, the majority of the genes with a known function were upregulated (71%). These genes were mainly associated with metabolism (e.g. transport and metabolism of amino acids) and information storage and processing (transcription, translation, repair). At day 2, the known *E. coli* O157:H7 genes were mainly downregulated (65%) and were involved in among others carbohydrate transport, cell wall biogenesis and transcription. Upregulation of numerous *E. coli* O157:H7 genes associated with oxidative stress and antimicrobial resistance, including the iron-sulfur cluster and the multiple antibiotic resistance (*mar*) operon, could be observed, whereas the Shiga toxin virulence genes were downregulated. Our findings reveal that the pathogen actively interacts with the plant environment by adapting its metabolism and responding to oxidative stress. Consequently, the pathogen may have acquired enhanced resistance against oxidative stress and cross-protection against various other stresses. On the other hand, a decrease in the expression of the Shiga toxin virulence genes was observed. Further research is needed to investigate how these adaptations may affect the pathogen's subsequent survival during processing and consumption.

6.1 Introduction

Leafy vegetables, such as lettuce, are considered as high risk food since numerous outbreaks with enteric pathogens have been linked to consumption of these produce (Klein *et al.* 2009). *Escherichia coli* O157:H7 (*E. coli* O157:H7) is one of the pathogens that is frequently involved and is of special interest due to the severe consequences of the illness it may cause. Infection may lead to bloody diarrhea, and occasionally to kidney failure. Especially young children, elderly and

immunocompromized persons are at higher risk of severe illness. The bacterium can enter the agricultural environment via animal feces and is able to enter our food chain from this point by e.g. contaminating the irrigation water used for growing crops, the use of untreated or non sufficiently treated manure (see also 1.5). A study found that more than 20% of all reported *E. coli* O157:H7 outbreaks in the United States were associated with fruits and vegetables, and lettuce was the single most frequently implicated commodity (Rangel *et al.* 2005). As governments promote the consumption of a wide variety of fresh fruit and vegetables, it is important that there is no increased risk for foodborne infections. Therefore, the understanding of plant-pathogen interactions such as initial adherence, invasion and establishment is essential for the development of effective control measures.

Numerous studies have investigated the factors which may influence the preharvest survival, growth and attachment of *E. coli* O157:H7 on growing plants. Mainly the influence of temperature, relative humidity, irrigation treatment, UV-radiation, leaf age, crop stage, crop variety, etc. were investigated. However, less is known about the underlying genetic mechanisms that *E. coli* O157:H7 uses to survive and proliferate on plants. On the plant surface, the pathogen may pass through long-term stresses that are variable in nature and complexity. In contrast to the intestines for example, nutrient scarcity is likely to occur, especially within the phyllosphere. This may enhance resistance of the pathogen to physical or chemical challenges and is thus highly relevant to the pathogens subsequent behavior during processing and storage of the produce but is not yet intensively investigated (Delaquis *et al.* 2007).

Gene expression profiling such as microarray technology is one of the techniques used to determine which processes are involved in the pathogen response to specific environmental variables. Only a few microarray studies were performed on the interaction between *E. coli* O157:H7 and fresh produce. Two studies mainly focused on post-harvest contamination. Kyle *et al.* (2010) have investigated the short term effects of the influence of leaf injuries and damaged leaf tissue on the gene expression of *E. coli* O157:H7 EDL933 on romaine lettuce leaf lysate. Their experiments revealed that the available carbohydrates from damaged tissue, activates the carbohydrate transport systems in *E. coli* O157:H7. Furthermore, an upregulation of *E. coli* O157's genes associated with attachment, virulence, oxidative stress, antimicrobial resistance, detoxification of noxious compounds and DNA repair but also an increased resistance to hydrogen peroxide and calcium hypochlorite could be observed (Kyle *et al.* 2010). Fink *et al.* (2012) have performed a study on the gene expression of pathogenic and generic *E. coli* on harvested, intact lettuce leaves. They have studied the midlong term effect (1-3 days) on surface sterilized leaves and observed differences in expression between generic *E. coli* K12 and pathogenic *E. coli* O157:H7 EDL933. Genes involved in

energy metabolism were downregulated whereas genes involved in biofilm modulation and curli production were significantly upregulated in *E. coli* K12 and O157:H7 (Fink *et al.* 2012). Preharvest contamination conditions were also investigated for *E. coli* K12 and O157:H7 interacting with the lettuce rhizosphere (Hou *et al.* 2012; Hou *et al.* 2013) but not with the phyllosphere, whereas this is important as this is the part of the crop that is consumed. Only recently also a report on the gene expression of *E. coli* O157:H7 on radish sprouts was published (Landstorfer *et al.* 2014).

The present study, investigates the gene expression of *E. coli* O157:H7 Sakai while interacting with the phyllosphere of growing butterhead lettuce plants after one hour and 2 days. Special attention was paid to the pre-inoculation conditions.

6.2 Material and methods

6.2.1 Bacterial strains and culture conditions

E. coli O157:H7 Sakai strain RIMD 0509952 (Sakai; Stx- Kanr) was used and was previously described by Dahan *et al.* (2004). The Sakai strain was isolated from the Sakai city outbreak in Japan in 1996 (Michino *et al.* 1999; Watanabe *et al.* 1999) and possesses a double *stx* knockout. A kanamycin cassette was inserted into the *SmaI* site in the *stx2A* gene, and a 0.6-kb BsiWI fragment, which contained the *stx1A* gene and the upstream region, was deleted (Dahan *et al.* 2004). Cells were pre-cultured in 5 ml LB and grown overnight at 37°C, 200 rpm. This culture was 1/100 diluted in pre-tempered enriched MOPS and grown at 18°C, 200 rpm for 22h to the early stationary phase of growth. The OD_{600} was measured (Genesys 10uv, Thermo Scientific) and the culture was washed with sterile 10mM $MgSO_4$ (230291, Sigma-Aldrich, S. Louis, MO, USA) (10 min, 5000×g, 4°C), and re-suspended in sterile 10mM $MgSO_4$ to an OD_{600} of 0.5 which corresponded with 8.7 ± 0.1 log CFU/ml as determined by plate counting on the selective medium sorbitol MacConkey Agar (SMAC, Sigma-Aldrich).

6.2.2 Plant growth conditions

Pelletized butterhead lettuce seeds (*Lactuca sativa* L. var. *capitata* 'Alexandria') were obtained from Rijk Zwaan Distribution B.V., De Lier, the Netherlands. The seeds were sown in a mixture of peat, sand, limestone, perlite, celcote, sincrostart and multicote-4. One week after sowing, the seedlings were placed in square pots of 10 cm and grown in the greenhouse at The James Hutton Institute (Dundee, UK). Four-week old plants were moved from the greenhouse to the growth chamber (Microclima1000, Snijders, Tilburg, The Netherlands) one day before the start of each experiment. Growth chamber conditions were set at continuously ± 18°C with a relative humidity (RH) of 75% and 16/8 hour light/dark cycle.

6.2.3 Inoculation of the lettuce plants and measurement of pathogen populations on lettuce leaves

Four-week old lettuce plants (± 9-leaf stage) were spray-inoculated in a biosafety cabinet. A total of 100 ml inoculum was used to inoculate 16 plants. The pathogen population on the leaves was determined in duplicate one hour after inoculation and 1, 2 and 3 days thereafter. For each sample, 1 g of leaves from 3 lettuce plants was used. The sample was ground aseptically with sterilized sand and 2 ml phosphate buffered saline (PBS), using a pestle and mortar. Serial dilutions were made in PBS and the appropriate dilutions were plated onto SMAC. Maceration does not recovering all of the interacting bacteria, but it is still one of the best methods for direct plate counts (Kisluk *et al.* 2012).

6.2.4 Determining the amount of loosely and strongly associated *E. coli* O157:H7 Sakai cells with lettuce

To determine the amount of loosely associated pathogens, 10 g lettuce leaves from 3 lettuce plants were removed with sterile forceps and placed in a sterile beaker with 200 ml sterile PBS. The lettuce was cut into pieces of ± 2cm^2 with sterile scissors and stirred on a magnetic stirrer for 5 min at level 3 (Stuart stir UC151). This treatment will be subsequently called 'gentle wash'. One ml wash water (1 ml) was taken and the appropriate serial dilutions were immediately plated onto SMAC agar. The wash water was decanted and the lettuce was rinsed with 200 ml PBS, poured into a sieve and allowed to drip. Then, after adding the lettuce to a fresh beaker, 200 ml PBS and 5 g of sterile glass beads with a diameter of 2-3mm were added. The lettuce was stirred for 10 min at level 5 and wash water samples were taken to determine the amount of bacteria that were removed (termed 'strong wash'). The beads were added in order to remove the upper cell layers of the lettuce leaves, so that the strongly associated and/or some of the internalized bacteria could be removed. The experiment was conducted one hour and two days after inoculation in triplicate and was repeated twice. The optimal wash protocol to remove the loosely (gentle wash) and strongly associated pathogens (strong wash) was determined in a preliminary test (data not shown).

6.2.5 RNA-extraction

Samples of 10 g of lettuce from different leaves from 3 lettuce plants was removed with sterile forceps. The leaves were prepared, washed (both gentle and strong wash) and rinsed as described above, but 200 ml ice-cold RNA-stop wash solution (0.5% phenol pH 4.3, 9.5% ethanol absolute and 90% PBS) was used instead of PBS. The RNA-extraction was performed on the wash water from both washes separately. For each wash treatment, the 200 ml RNA-wash solution was pipetted into four 50 ml RNAse free polypropylene tubes and centrifuged for 10 min, 4°C, 5000 rpm. The supernatant was removed and the pellets were dissolved in 1 ml ice-cold RNA-stop (5% phenol pH4.3, 95%

ethanol absolute). These solutions were collected in a 50 ml tube. Subsequently, the empty tubes were rinsed with another 1ml of ice-cold RNA-stop and this was also added to the 50 ml tube and centrifuged as described above (5 ml in total). The supernatants was removed and the pellet was once more washed with 1 ml ice-cold RNA-stop solution. The pellet was stored at -80°C. The bacterial pellets were treated with 50 mg/mL of lysozyme (5 min) (Sigma Alrich, L3790). Subsequently, RNA was extracted with the Qiagen RNeasy kit with an additional on-column DNase digestion performed following the instructions of the manufacturer (Qiagen, Hilden, Germany). A cell pellet of 1 ml of the inoculated $MgSO_4$ buffer was made one hour after inoculation following the Qiagen RNeasy protocol and also stored at -80°C. RNA-extraction was performed as described for the lettuce wash water samples. Total RNA was quantified spectrophotometrically (NanoDrop ND 1000, Thermo Scientific, Wilmington, DE) and the quality examined with a Bioanalyzer 2100 (Agilent technologies, Palo Alto, CA). The RNA was immediately stored at -80°C.

6.2.6 Microarray labelling procedure

A volume of 17.7 µl of total RNA was combined with Enterobacteriaceae specific 10 x 11-mer oligos, 100ng/µl as described by Fislage *et al.* (1997) on ice, incubated 10 min at 70°C and cooled on ice again. 17.3 µl of a master mix (5x first strand buffer, 9.0 µl; 0.1M DTT, 4.5 µl, 25x aa-dNTP labelling mix 1.8µl Superscript RT 2.0µl) was added and the samples were incubated for 2 h at 42°C. 15µL of 1M NaOH and 15 µl 0.5M EDTA (pH 8.0) was added to hydrolyse the RNA and the samples were incubated for 15 min at 65°C. Then 15 µl 1M HCl was added to neutralize the samples. cDNA was purified by adding 450µl PB buffer (Qiagen) mix and applied to a Qiagen MinElute column. The samples were centrifuged. Centrifugation always occurred at 13 000 rpm for 1 min unless stated otherwise. The supernatans was removed and 750 µl filter sterilized phosphate wash buffer (0.25ml 1M phosphate buffer, 7.7 ml SDW, 42.2 ml absolute EtOH) was added and centrifuged. The supernatant was removed and the column spun for 2 min at full speed. The column was transferred to a new amber 1.5 ml tube and 10 µl filter sterilized phosphate elution buffer (0.2 ml 1M phosphate buffer, 49.8 ml SDW) was added to the center of the membrane, left for one minute at room temperature and subsequently centrifuged. This step was repeated with another 10 µl filter sterilized phosphate elution buffer. The subsequent reactions were performed in low light and the incubations were performed in the dark. Sodium carbonate buffer (2.0 µl, 1M) was added to the purified cDNA and mixed. Subsequently, 1.0 µl of the appropriate Cy-dye (GE Healthcare #PA23001, PA25001) (in DMSO) was added and incubated for 1 h at room temperature in the dark. The labeled cDNA was purified by adding 3.0µl 4.0M hydroxylamine hydrochloride, was mixed and incubate in dark for 30 min. The volume of each reaction was made up to 100 µl with SDW. 500 µl PB buffer was added, mixed and applied to a Qiagen MinElute column and centrifuged. The supernatant was removed and

750 µl PE buffer was added and then centrifuged. The supernatant was removed and the tube was once more centrifuged. The column was transferred to a new 1.5 ml tube and 10 µl elution buffer was added to the center of the membrane and left for 1 min at room temperature and then centrifuged. The sample was re-eluted with an additional 10 µl elution buffer into the same tube. The Cy3/Cy5 incorporation was estimated by NanoDrop ND 1000.

6.2.7 Preparation of prokaryotic hybridization samples for Agilent 8 x 15K Arrays

The volume of each labeled cDNA was calculated to give 600 ng and pipetted into a fresh 1.5 ml amber tube. Nuclease free water (Sigma-Aldrich) was added up to 20 µl. A volume of 5 µl of Agilent 10 x Blocking Agent was added to each tube and the samples were denatured at 98°C for 3 minutes and cooled to room temperature. 25 µl of 2 x GEx Hybridisation Buffer HI-RPM was added to each tube, mixed well by careful pipetting. The tubes were spun for 1 minute at room temperature at 13 000 rpm and immediately placed on ice and loaded onto the array. Forty microliter of each mix was added to the gasket slide and the Agilent array slide was placed on top. The slides were incubated for 17 hours at 65°C with rotation at 10 rpm in a hybridization oven. Three biological replicates were prepared for each time point (1 h and 2 days after inoculation).

6.2.8 Uninoculated lettuce control

To check whether the microbial background microbiota on the lettuce may interfere with the micro-array spots, uninoculated samples were checked for the presence of the *gadA* gene, a housekeeping gene of *E. coli*. The presence of *gadA* was checked by conventional PCR. The forward primers was 5' ACCTGCGTTGCGTAAATA and the reverse 5' GGGCGGGAGAAGTTGATG and were described in (Kim *et al.* 2006). Each PCR reaction consisted of 5 µl GoTaq Buffer Green, 3 µl dNTPs (2.5 mM), 0.2 µl 50 µM forward primer, 0.2 µl 50 µM reverse primer, 0.5 µl cDNA, 0.2 µl polymerase and 15.9 µl SDW. The reaction mixture was processed in an thermocycler (TProfessional basic Thermocycler gradient, Biometra) with the following settings: 2 min at 94°C, 30 cycles: 30 s at 95°C, 30 s at 56.7°C, and 1 min at 72°C , followed by a final extension time at 72°C for 7 min. 5 µl of PCR-product was loaded on a 1% agarose gel and imaged under UV illumination. The *gadA* gene was not detected on uninoculated lettuce.

6.2.9 Data analysis

The data were analyzed using the software Genespring version 7.0 (Agilent/Stratagene). The data from all four biological replicates were further tested by a Principal Component Analysis (PCA) on the

different conditions (mean centering and scaling). The outliers were removed by filtering on flags (present or marginal). The data were normalized to the control samples and the replicates combined.

Genes showing a greater than 2-fold upregulation or downregulation following Volcano Plot analysis ($0.01 \leq P < 0.05$ or $P < 0.01$) and a raw expression value > 50 were considered to be differentially regulated. Comparison of the expression levels was done for the expression after 1 hour (day 0) with the inoculated $MgSO_4$ buffer, day 2 with the inoculated $MgSO_4$ buffer and day 2 in comparison with 1 hour after inoculation (day 0). A False Discovery Rate (FDR) was not conducted at these significance levels.

Subsequently, a single gene list was made as the Agilent array slide contained the genes of 4 different *E. coli* strains: the core genome of generic *E. coli* K12-MG1655, strain specific genes of of *E. coli* O157:H7 EDL933, *E. coli* O157:H7 VT2-Sakai, and uropathgenic *E. coli* CFT073. Genes of uropathogenic *E. coli* CFT073 were not taken into consideration. Furthermore, gene sequences of all significantly upregulated *E. coli* K12, *E. coli* O157:H7 EDL933 and *E. coli* O157:H7 VT2-Sakai without a gene name were looked up on XBase, when duplicates were present in the database, the *E. coli* Sakai gene was kept if possible, if duplicates were present between *E. coli* K12 and *E. coli* O157:H7 EDL933, the *E. coli* K12 gene was kept. Unknown *E. coli* O157:H7 EDL933 were discarded from the list as well. This single gene list was used to link the genes with a COG-annotation (https://img.jgi.doe.gov). Furthermore, gene lists were made based on the GenProtEC Multifun classes. Therefore, all K12-genes and *E. coli* Sakai genes with a gene name that similar to K12 were linked to the following classes: class 5 cell processes, class 6.3 pilus, 6.4 flagella and 6.6 ribosomes) (http://genprotec.mbl.edu/).

For the virulence gene list, *E. coli* O157:H7 Sakai virulence genes as described in Hayashi *et al.* were taken into account and a keyword search was performed on the GIRC-site (http://genome.bio.titech.ac.jp/bacteria/o157/search.html) (Hayashi *et al.* 2001). The keywords that were used were: invasion, adhesion, fimbria, effacement, toxin, type III secretion. For each gene list, the relative expression levels on day 0 and day 2 were plotted against the $MgSO_4$ control and significantly regulated genes were highlighted using Matlab.

The nucleotide sequence of the significantly regulated unknown genes with and ECs number was obtained by XBase search. This nucleotide sequence was blasted in Blast () in order to find out if the gene was already known, described in related strains.

6.3 Results and discussion

In this study, the interaction of *E. coli* O157:H7 with intact growing young lettuce plants was investigated with conventional plate counting technique and microarray technique investigating the transcriptional changes in *E. coli* O157:H7. The results will be discussed and compared with the existing literature, in particular with two gene expression studies that are closely related with this study and were already described in the introduction. An overview of the main differences between the different studies is shown in Table 6.1 (Kyle *et al.* 2010; Fink *et al.* 2012).

Table 6.1: Overview of the main differences between the different gene expression studies with *E. coli O157:H7* and fresh produce.

	Kyle *et al.* 2010	**Fink *et al.* 2012**	**This study**
Strain	*E. coli* O157:H7 EDL933	*E. coli* K12 MG1655 *E. coli* O157:H7 EDL933	*E. coli* O157:H7 Sakai
Growth medium	M9-glucose	LB	MOPS-enriched
Growth temperature *E. coli*	28°C	37°C	18°C
Time points	15 min; 30 min	1 and 3 days	1 h, 2 days
Lettuce type	romaine lettuce	green leaf lettuce	butterhead lettuce
Treatment	leaf lysate supernatant	surface sterilized leaves sodium hypochlorite	growing plants
Incubation conditions		100% RH, 25°C photoperiod 16 h for 3 days.	Growth chamber, 18°C , 75% RH, photoperiod 16 h for 2 days.
Reference control	M9-glucose, 28°C	LB, 37°C	$MgSO_4$ buffer, 18°C

6.3.1 Survival and association of *E. coli* Sakai on/with growing butterhead lettuce

The survival of *E. coli* O157:H7 Sakai on young butterhead lettuce during 3 days is shown in Figure 6.1 A. The pathogen was inoculated at a level of 7.45 ± 0.37 log CFU/g lettuce. This level dropped to 5.02 ± 0.27 log CFU/g after 2 days and the pathogen level did not significantly change anymore at day 3.

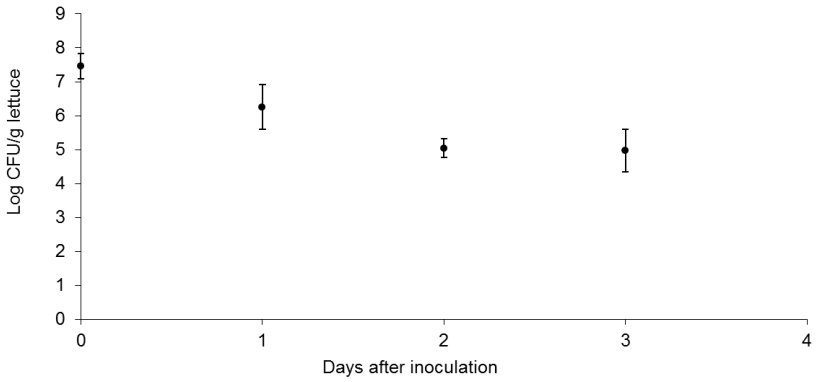
The association of the pathogen to the lettuce was analyzed one hour and two days after inoculation. Two different washing steps were performed (gentle wash and strong wash), the results of the efficiency of these washing steps are shown in Figure 6.1 B. One hour after inoculation, around 90% of the pathogens could be removed from the lettuce with the gentle wash protocol (± 1 log reduction on the lettuce), and the subsequent 'strong wash' could remove another 90% of the pathogens (± 1 log reduction). Two days after inoculation of the lettuce, similar results could be seen, although higher variation was observed. From the graphs it can be seen that, based on the selective plate

counts, the amount of pathogens that could be removed from the lettuce at day 0 with the strong wash protocol was similar to the amount of pathogens that could be removed from the lettuce at day 2 with the gentle wash protocol. For the RNA-extraction, however, only RNA extracted from the gentle wash at day 2 contained enough bacterial RNA to be acceptable to perform the microarray. Therefore, the microarrays were only performed on the loosely associated (removed by the gentle wash) and not on the strongly associated bacteria. These results may be caused by technical difficulties as the matrix of the strongly associated bacteria contained a lot of lettuce material, which reduced the amount of bacterial RNA that could be extracted. Furthermore, the results may also indicate that at day 2 a part of the pathogen population was still alive and metabolically active, but not able to grow anymore on selective culture medium. Similar findings were found by Moyne *et al.* (2013). In their growth chamber studies, viable *E. coli* O157:H7 quantities determined by PMA real-time PCR were approximately 10^4 greater than found by colony enumeration one hour after introduction onto lettuce plants (30% RH). Also Dinu and Bach (2011) have shown that the viable but non culturable (VBNC) response can be induced in *E. coli* O157:H7 when introduced on lettuce leaves.

6.3.2 Transcriptome of *E. coli* O157:H7 Sakai on growing butterhead lettuce

A total of 273 genes (5.04%) of the Sakai genome were induced or repressed by at least twofold ($P<0.01$, raw value > 50 for all treatments) in comparison with the inoculated simulated contaminated irrigation water ($MgSO_4$ buffer). There was a clear difference between the functions of the genes that were differently regulated after being on the lettuce for one hour and after two days (Figure 6.2). One hour after inoculation, 164 of the selected genes were significantly differentially regulated, with the majority of the genes induced (71%). The upregulated genes that could be assigned to a category of orthologous genes (COG), belonged mainly to transport and metabolism of amino acids and inorganic ions on the one hand and transcription, translation, ribosomal structure and biogenesis on the other hand. At day 2, 147 genes were significantly differentially regulated with the majority of the selected genes (65%) downregulated. *E. coli* O157:H7 genes that were downregulated included those involved in carbohydrate transport and metabolism, cell wall/membrane, envelope biogenesis, and transcription. Only 37 of the selected genes were significantly up or downregulated at both time points. For almost 40% (39.9%) of the selected genes, a COG class could not be assigned or the genes were assigned as poorly characterized or function unknown. It is also noteworthy that 23.8 % of the selected genes were *E. coli* O157:H7 Sakai specific genes for which only an ECs number and no gene name was assigned.

A



B

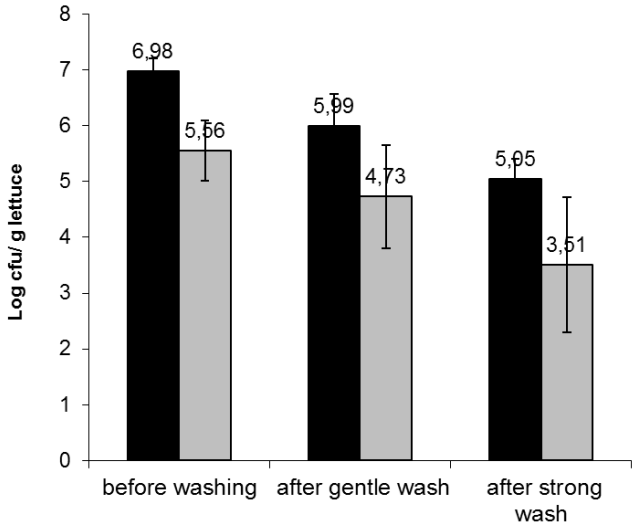
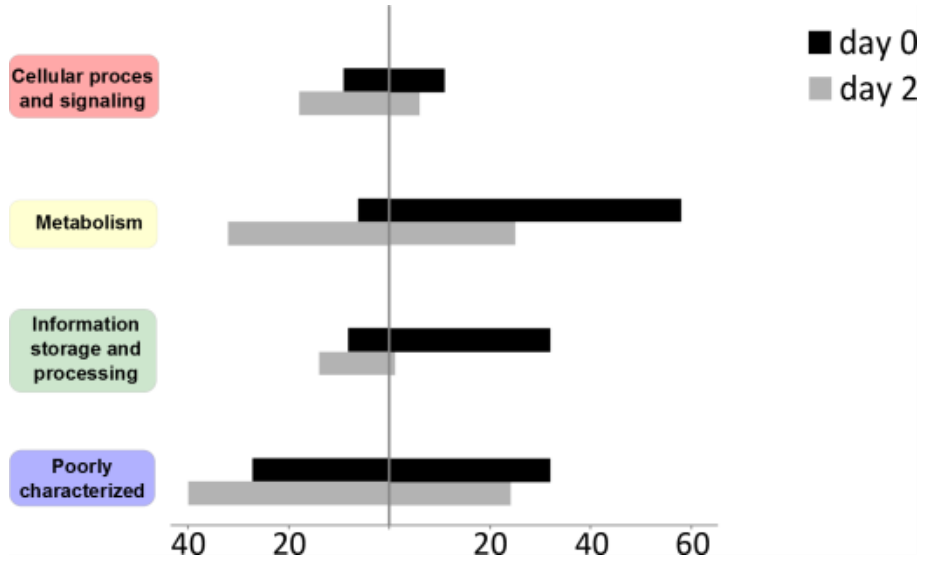


Figure 6.1: A. Survival of *E. coli* O157:H7 Sakai on butterhead lettuce. Triplicate samples from 2 independent experiments. B. Reduction of *E. coli* O157:H7 Sakai level 1 hour (black) and 2 days (grey) after inoculation on butterhead lettuce after performing gentle and strong wash protocol.

A



B

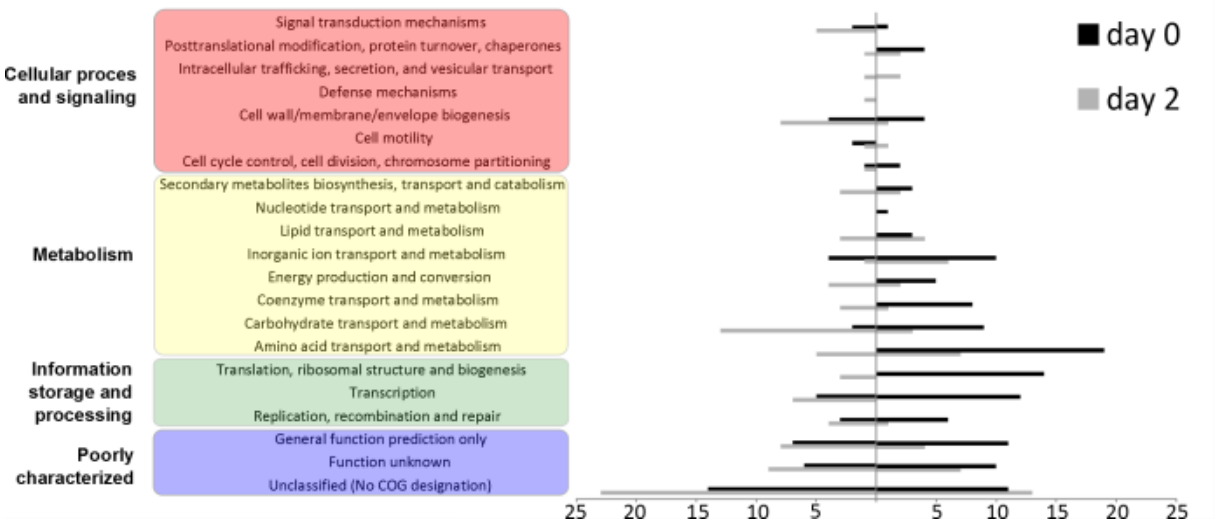


Figure 6.2: Overview of the significantly regulated genes (P<0.01, at least 2-fold, raw value >50), classified by COG group (A) and COG functional category (B)

6.3.3 Transcription and translation

(Figure 6.3, Figure 6.4). Different signals points towards an attempt of the pathogens to proliferate on the lettuce one hour after inoculation. For example, the upregulation of the ribosomal related genes such as the 30S (e.g *rpsU* at P<0.01 and rpsJT at P<0.05) and 50S ribosomal subunit proteins (*rplCM* at P<0.05). Furthermore, cell division related genes such as *dacA*, *groS* (P<0.01) and *mnmG* (P<0.05) were significantly upregulated one hour after inoculation. Also other genes related to transcription and translation such as *fis* which codes for a DNA-protein which is characteristic for cells in the log-phase (Ryan *et al.* 2004), transcripts coding for among others RNA-helicases (*rhlE*),

translation initiation factor IF-1 (*infA*), transcription termination (*rho*), and genes responsible for rRNA modification such as methylation of nucleotides (*mnmA*, *rumB*, *ECs4154*) were significantly upregulated one hour after inoculation. In contrast, *ftsN*, an essential cell division protein, was significantly downregulated. At day 2, the attempt to proliferation could not be observed anymore. Significant downregulation could be seen for all the ribosomal subunit proteins which were significantly upregulated one hour after inoculation (*rpsUJT*, *rplCM* at P<0.05, compared to their expression level one hour after inoculation) and for other ribosomal subunit proteins (*rpsFP* at P<0.01, *rpsDIKT*, *rplKUXY* at P<0.05). *ftsN* was further downregulated as well. These results are in agreement with the results from the plate counts for which we saw a decrease of the amount of culturable pathogens. Also Fink *et al.* 2011 observed a decrease in the expression of the *E. coli* O157:H7 ribosomal related genes one and three days after inoculation onto detached lettuce leaves.

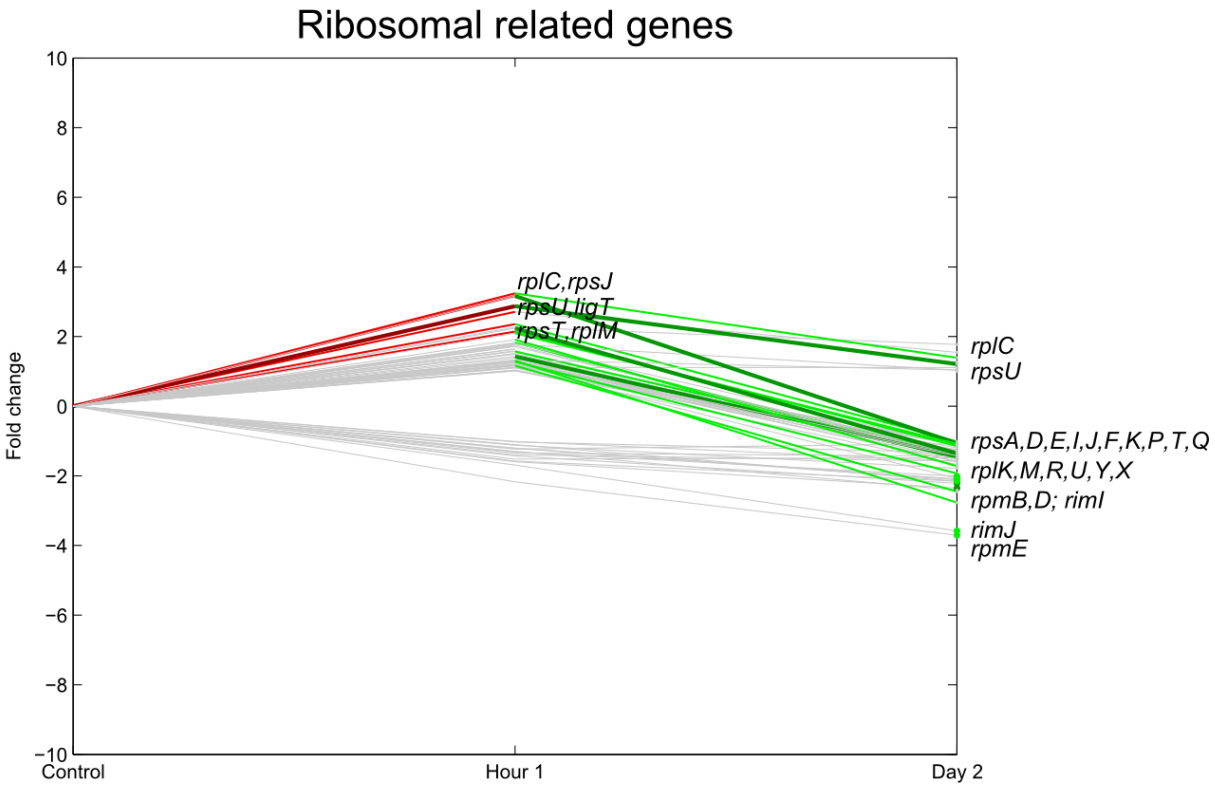


Figure 6.3: Relative expression of *E. coli* O157:H7 Sakai ribosomal related genes one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at 0.01≤P<0.05, dark green: downregulation at P<0.01, light red: upregulation at 0.01≤P<0.05, dark red: upregulation at P<0.01. Grey: no significant difference in expression. Data represent the mean of four biological replicates.

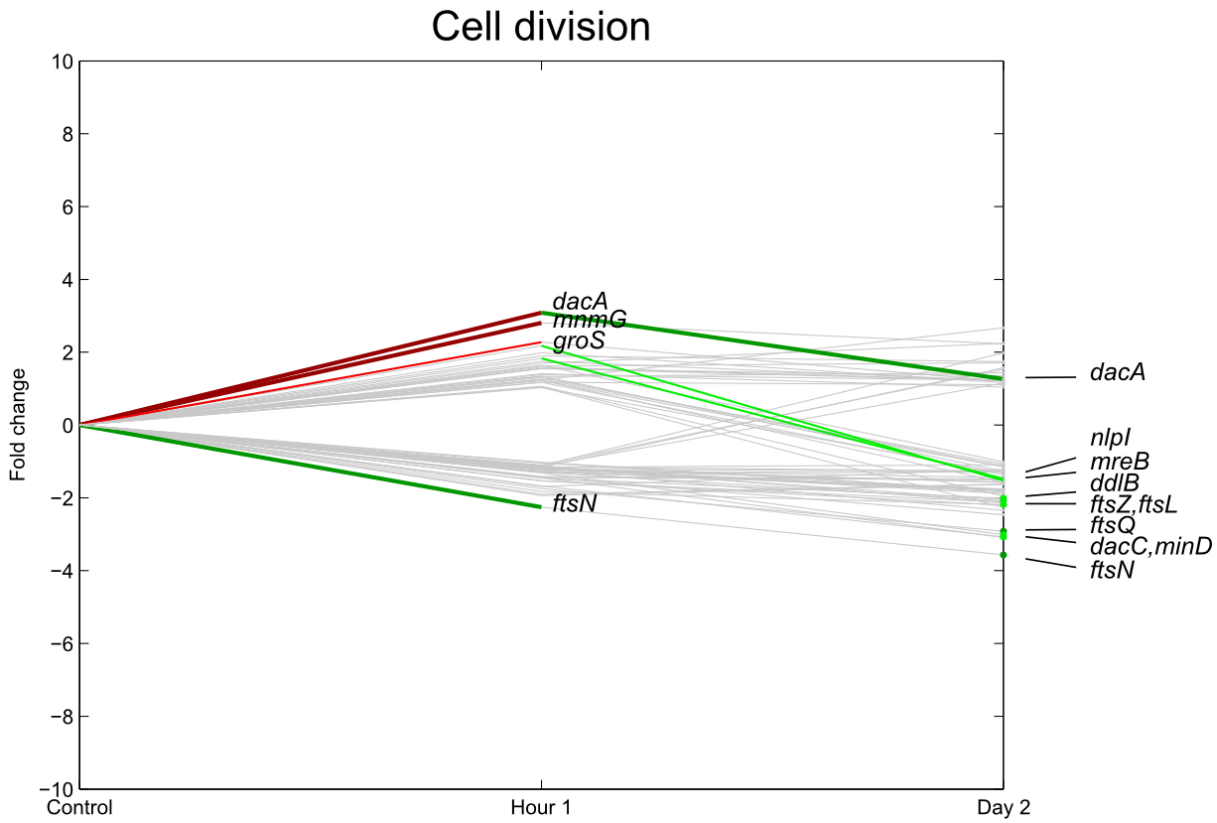


Figure 6.4: Relative expression of *E. coli* O157:H7 Sakai genes related with cell division one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at $0.01 \leq P < 0.05$, dark green: downregulation at $P < 0.01$, light red: upregulation at $0.01 \leq P < 0.05$, dark red: upregulation at $P < 0.01$. Grey: no significant difference in expression. Data represent the mean of four biological replicates.

6.3.4 Stress responsive genes

(Figure 6.5, Figure 6.6). Almost one third of the upregulated genes belonging to the COG-classes amino acid or inorganic ion transport and metabolism were also known to be stress responsive genes, some of these genes are also related with drug resistance.

A first set of genes that was significantly upregulated one hour after inoculation were genes involved in Fe-S cluster assembly and repair, i. e. the ironsulfur cluster (*iscSRUA-hscBA-fdx*). These genes are essential for all proteins containing Fe-S clusters which participate in many vital cellular function such as DNA repair, transcriptional regulation, nucleotide and amine acid biosynthesis and energy metabolism (Schwartz *et al.* 2000). The iron sulfur cluster, mobilizes Fe and S atoms, which are both toxic for the cell in soluble form in the cytosol, assemble them to Fe-S clusters and transport them without creating toxic conditions (Fontecave *et al.* 2005). Contrarily, two other systems with a similar function, the *suf*-system (sulfur assimilation) and the *csd*-system (cysteine sulfinate desulfinase),

were not differentially regulated in our experiment. Expression of the three Fe-S cluster assembly operons is regulated by oxidative stress as Fe-S clusters are very sensitive for active oxygen species and iron limitation (Schwartz *et al.* 2000; Hidese *et al.* 2011).

Based on our data, there is no indication for iron limitation, as the *feb* operon (*febA*) related to iron transport was not differentially upregulated. But sulfur depletion may have occurred as the cysteine operon (*cys*) was upregulated at both one hour and two days after inoculation. Cysteine is one of the twenty natural amino acids. Together with methionine it is the only amino acid containing sulfur, therefore, it serves as a precursor for many forms of reduced organic sulfur which is present in cofactors such as thiamin and Fe-S clusters (Hidese *et al.* 2011). Also some genes of the *tau* operon, which are related to sulfur acquisition were significantly upregulated at either day 0 (*tauB* at day 0 and day 2, $P<0.05$) or day 2 (*tauA*, $P<0.01$). Similar results were observed by Fink *et al.* , they also saw that genes involved in Fe-S cluster assembly and repair (*isc*) were upregulated, whereas similar to our experiment, the two other Fe-S cluster related operons *suf* and *csdAE* operon were not differentially expressed.

Furthermore, increased expression of several genes involved in multi drug resistance (MDR) and response to inhibitory compounds was observed. The *marRAB* operon was consistently upregulated in the pathogen one hour and two days after inoculation, with a higher and significant upregulation one hour after inoculation. *marA*, multiple antibiotic resistance gene A, is one of the transcription factors which coordinates the multiple mechanisms that bacteria possess to survive exposure to various chemical stresses and antimicrobial compounds. Induction of *marA* may result in increased resistance against a variety of antibiotics such as tetracycline, chloramphenicol, penicillins, cephalosporins, rifampin, puromycin, nalidixic acid, fluoroquinolones and oxidative stress (Cohen et al. 1993, Wang *et al.* 2009). The transcription of the *marRAB* operon is regulated by *marR* in response to a wide variety of compounds such as antibiotics, oxidizing but also to phenolic compounds. Furthermore, it has been postulated that plant derived naphthoquinones might be natural inducers of *marA* (Miller and Sulavik 1996 in Randall and Woodward 2002). MarA is an MDR efflux pump. Interestingly, different observations suggest a role of MDR pumps in plant/bacteria interactions, apart from their role in processes of detoxification of intracellular metabolites, bacterial virulence in both animal and plant hosts, cell homeostasis and intercellular signal trafficking (Martinez *et al.* 2009). First, the organisms with the largest number of MDR pumps are found in the soil or in association with plants (Konstantinidis and Tiedje 2004). Second, Matilla *et al.* (2007), observed that several efflux pumps are induced when *P. putida* adjusts its genetic program to the colonization of roots. Another example is AcrAB-TolC, the most important MDR pump of Enterobacteriaceae. In *E. coli*, expression of this efflux pump is controlled by the quorum sensing regulator SdiA and studies

suggest that this pump can efflux *E. coli* autoinducers, thus contributing to the quorum signal response (Rahmati *et al.* 2002; Yang *et al.* 2006).

Also other genes, involved in the degradation of inhibitory phenolic compounds such as azoreductases *azoR* and *acpD*, were highly upregulated (Liu *et al.* 2009, Goudeau *et al.* 2013). *AzoR* was also upregulated in *Salmonella* when it came in contact with soft rot (Goudeau *et al.* 2013) and was recently reported to be upregulated on radish sprouts (Landstorfer *et al.* 2014). These last authors speculate on a role of this enzyme in detoxification of secondary plant metabolites directed against, or modulating, the bacterial microbiome.

Phenolic compounds such as salicylic acid (SA) can be produced by the plant, these molecules play together with jasmonic acid (JA) an important role in protecting the plant from further infection after an initial pathogen attack or small injuries. It was already shown on different plants (*Arabidopsis, Medicago, Tobacco*) that the presence of *E. coli* O157:H7 or *Salmonella* could induce a SA-dependent response in the plant (Schikora *et al.* 2008; Shirron and Yaron 2011; Seo and Matthews 2012; Roy *et al.* 2013; Jayaraman *et al.* 2014). Curli and flagella were recognized by the plant defense system and a common set of genes was induced in the plant by *E. coli* O157:H7 and *Salmonella* (Jayaraman *et al.* 2014). Induction of the plant defense system as in response to the presence of *E. coli* O157:H7 Sakai may be the cause of the upregulation of the above mentioned genes, although it cannot be excluded as well that unintentionally made small injuries made during transport and handling of the plant possibly could have induced the plant defense response as well, although this was prevented as much as possible. Upregulation of the *mar* operon was also described by both Fink *et al.* when *E. coli* was inoculated on detached lettuce leaves and by Kyle *et al.* where *E. coli* O157:H7 was inoculated into lettuce lysate. However, Kyle *et al.* observed a much stronger upregulation due to damage of the lettuce tissue.

Two other homologous transcription factors, *soxS* and *rob* are known to regulates a common set of genes with *marA*. These three transcription factors are individually regulated by different systems in order to be able to tune the pathogens response to a specific stress, but they have also the potential to regulate each other's expression. In our experiment only the *mar* operon was significantly up regulated where *rob* and *soxS* were significantly downregulated (*rob*: day 2 ($P<0.05$), *soxS*: both 1 hour after inoculation (-2.6) and day 2 (-3.3)). Downregulation of *soxS* was also observed by Fink *et al.* and Kyle *et al.*

In general many other oxidative stress genes were upregulated as well (*grxB*, *yeeE*, *yjgH*, *yjgI*) although the transcripts of the stress related gene regulators *rpoS* and *relA* (stringent response) were

not significantly induced. Oxidative stress may have been caused by active oxygen species, a byproduct of normal aerobic metabolism of the lettuce plant.

Also *cspA*, cold shock protein A, was significantly upregulated the first hour after inoculation. This gene is the major cold shock protein of pathogenic *E. coli*. It is an RNA chaperone that binds to single-stranded RNAs to prevent misfolding due to formation of secondary structures at cold temperatures (Duffitt *et al.* 2011; Kortmann and Narberhaus 2012). Cold shock, however, was prevented as much as possible during our experiments as the MOPS inoculum, the $MgSO_4$ buffer and the temperature of the growth chamber were incubated or set at 18°C. A plausible explanation for the increase in cold shock protein expression is suggested by Lesley *et al.* who have shown that upregulation of cold shock proteins can also be an indication of paused translation as a response on the presence of misfolded proteins, a response that is independent of any temperature shifts (Lesley *et al.* 2002).

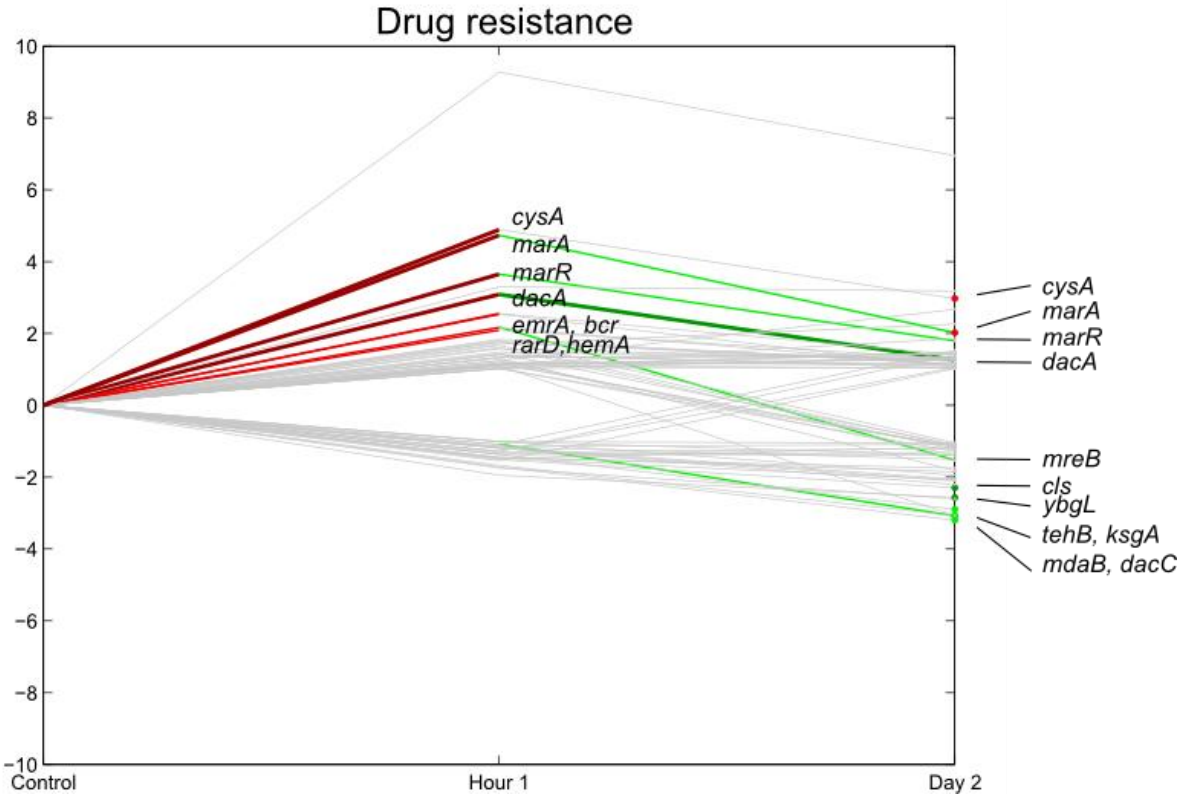


Figure 6.5: Relative expression of *E. coli* O157:H7 Sakai genes related with drug resistance one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at $0.01 \leq P < 0.05$, dark green: downregulation at $P < 0.01$, light red: upregulation at $0.01 \leq P < 0.05$, dark red: upregulation at $P < 0.01$. Grey: no significant difference in expression. Data represent the mean of four biological replicates.

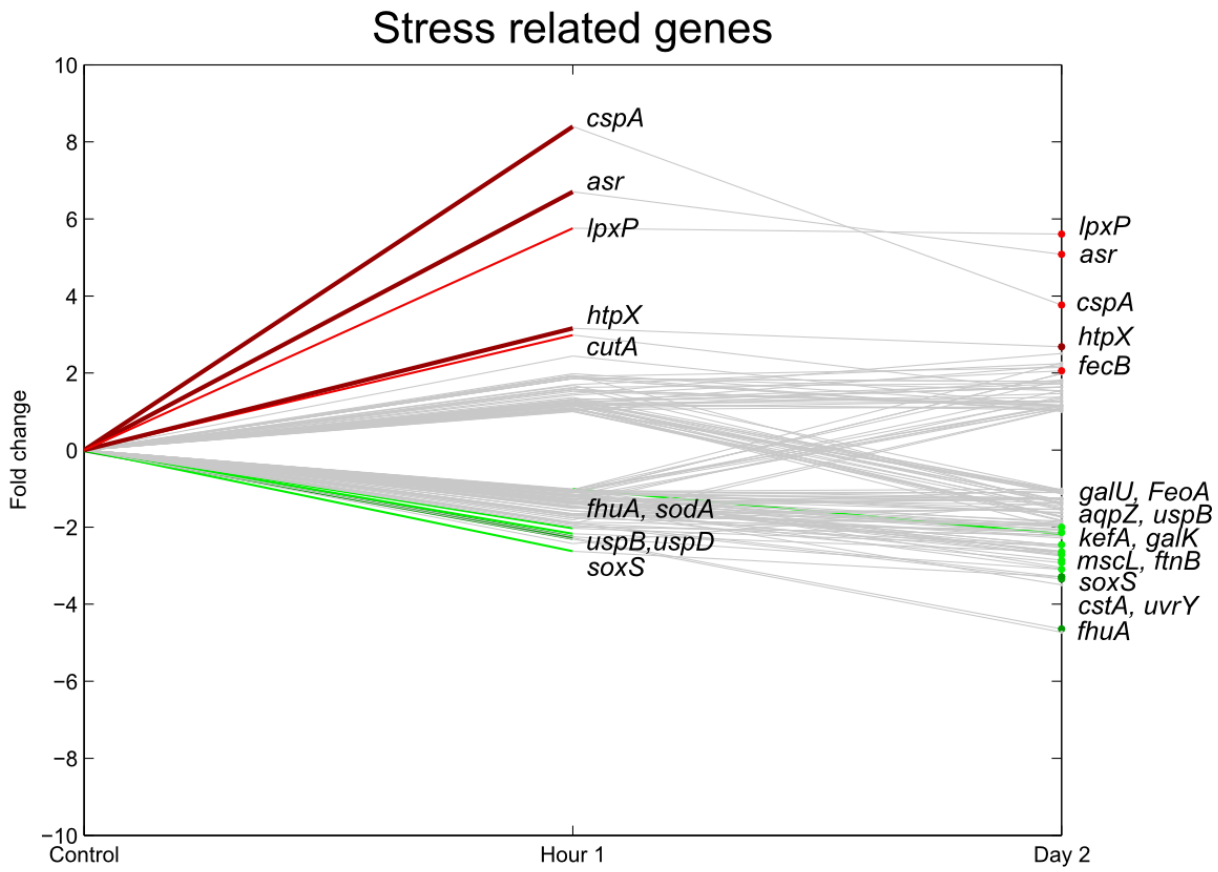


Figure 6.6: Relative expression of *E. coli* O157:H7 Sakai genes related with stress one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at $0.01 \leq P < 0.05$, dark green: downregulation at $P < 0.01$, light red: upregulation at $0.01 \leq P < 0.05$, dark red: upregulation at $P < 0.01$. Grey: no significant difference in expression. Data represent the mean of four biological replicates.

6.3.5 Carbohydrate transport

(Figure 6.7). One hour after inoculation, genes essential for the utilization of fructose as a carbon source (*fruB* and *fruK*) were significantly upregulated. *FruAB* takes up exogenous fructose, releasing the 1-phosphate ester into the cell cytoplasm in preparation for metabolism, primarily via glycolysis (EcoCyc 2014). Whereas *fruK*, phosphorylates fructose 6-phosphate during glycolysis (Anonymous 2014). The carbon starvation protein (*cstA*), which is induced by carbon starvation, was downregulated one hour and two days after inoculation.

The uptake of these sugars was only a short term effect as on day two the expression levels did not change significantly anymore further upregulation could only be seen for *alsC,* responsible for the uptake of D-allose. Several other carbohydrate transport related genes were downregulated two

days after inoculation together with other genes involved in (or predicted to be involved in) sugar transport and metabolism (*araG*, *araF*, *dhaL*, *dhaM*, *mak*, *manX*, *sfsA*, *xylH*, y*tfQ*, *ytfT*, *yjfF*, ECs5207).

Upregulation of the carbohydrate system may be explained by the fact that more nutrients are available on the lettuce leaves in comparison with the simulated irrigation water. Sucrose is a major translocatable product of photosynthesis and the main soluble component of the phloem sap, its monosaccharides are glucose and fructose. It was already shown that near the guard cells of leaves, up to 150 mM (Lemoine 2000; Kang *et al.* 2007 in Kroupitski *et al.* 2009) sucrose can be present. Moreover, for *Salmonella* it was shown that the pathogen is attracted to all three sugars and actively moves towards it on lettuce leaves (Kroupitski *et al.* 2009).

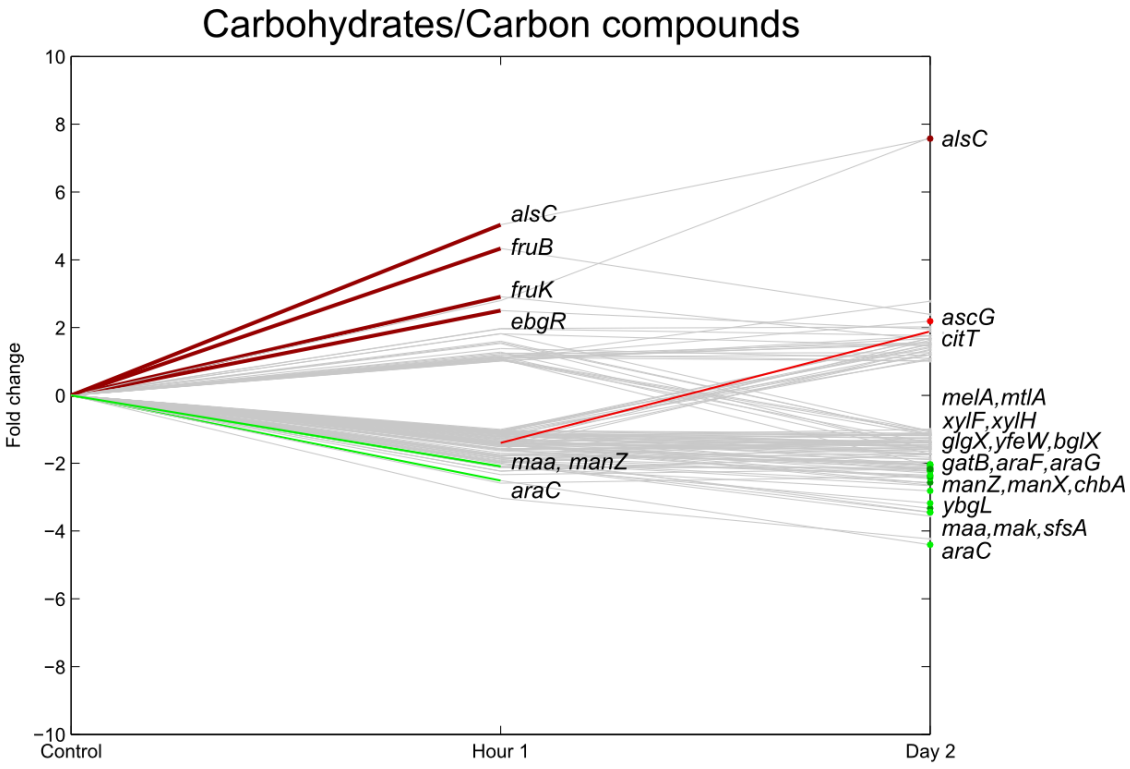


Figure 6.7: Relative expression of *E. coli* O157:H7 Sakai genes related with carbohydrate uptake one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at $0.01 \leq P < 0.05$, dark green: downregulation at $P < 0.01$, light red: upregulation at $0.01 \leq P < 0.05$, dark red: upregulation at $P < 0.01$. Grey: no significant difference in expression. Data represent the mean of four biological replicates.

6.3.6 Flagella, motility, fimbrial expression, biofilm

(Figure 6.8, Figure 6.9). Bacterial adherence to host tissues is a complex process and is dependent on difference factors: e.g. kind of bacterial strain/species, host tissue and environmental factors (Van der Linden *et al.* 2014). Several authors have already proposed that the presence of flagella and curli and the possibility of the bacteria to produce a biofilm may be involved in attachment of enteric pathogens to plant tissue.

Flagella are cellular appendages involved in the motility of the pathogen and were already related to be involved in the attachment mechanism of pathogenic *E. coli* to fresh produce (Xicohtencatl-Cortes *et al.* 2009). Also for *Salmonella* which is genetically closely related to *E. coli*, it was shown that the presence of flagella could increase the attachment capacity of the bacterium to plant tissue for some strains (Berger *et al.* 2009; Kroupitski *et al.* 2009). Furthermore, it was also shown that a mutant flagella deficient *Salmonella* strain showed reduced internalization into lettuce leaf tissue compared to its wildtype strain (Kroupitski *et al.* 2009).

In our study, only a few genes related to flagella, were significantly regulated, with the majority of these genes downregulated. *Tar, cheA, cheW,* and b0374, were significantly downregulated 2 days after inoculation. Only *fliN,* which is responsible for flagellar export, was significantly upregulated one hour after inoculation. Taken into account the reduction of cell counts two days after inoculation, these findings are in accordance with the results of Jozefzuc *et al.* and Lemuth *et al.* who observed downregulation of genes assigned to flagella and motility in response to glucose deprivation and a variety of stress conditions (cold, heat, oxidative stress, lactose diauxie, and stationary phase) (Lemuth *et al.* 2008; Jozefczuk *et al.* 2010). As flagella motility requires a steep proton gradient between the periplasmatic space and the cytoplasm, both authors hypotize that decreased cell motion could be an indication of energy deficiency. Also Fink *et al.* (2012), who investigated the gene expression of *E. coli* and *E. coli* O157:H7 on detached lettuce leaves, did not observe an induction of the flagellar genes (*fli* operon). Kyle *et al.*, however, observed an increase in *E. coli* O157:H7 *fli* genes in response to lettuce lysates, as compared to growth in M9 medium. In their experiment, however, nutrient replenishment occurred. This indicates that the influence of postharvest practices (e.g. presence of lettuce lysate due to wounding e.g. in fresh cut produce) can have a totally different influence on the gene expression of the pathogen.

Furthermore, flagellin, the protein that is the basic component of the filament of the flagella, is also known to be a major PAMP, recognized by plants a.o. (Thilmony *et al.* 2006). In order not to be recognized by the plant, downregulation of genes assigned to flagella may, therefore, be a reaction on the PAMP-triggered immunity response of the plant.

Also fimbrial expression has already been related to the attachment of enteric pathogens to fresh produce (Patel *et al.* 2011; Saldaña *et al.* 2011) but their importance seems again to be strain dependent (Boyer *et al.* 2007). Fimbria are filamentous appendages and are supposed to have an important role in the invasion of the animal intestine. Curli, a specific type of fimbria, are known to mediate binding to proteins and abiotic surfaces, and are also related to biofilm formation (Blomfield 2007). They were originally identified by their ability to mediate the binding of Congo red and they are composed of a major (*csgA*) and minor (*csgB*) unit and are expressed in most enterohemorrhagic and enterotoxigenic strains, but not in enteroinvasive and enteropathogenic strains (Van Houdt and Michiels 2005). In *E. coli* O157:H7 Sakai chromosome, a total of 14 loci encoding a set of genes for fimbrial biosynthesis were identified. Ten of these loci are also conserved or partially conserved in K-12, four are unique to *E. coli* O157:H7 Sakai (Hayashi *et al.* 2001).

In our study, similarly to flagella related genes, genes related to curli expression (operon *csgDEFG* and *csgBAC)* were in general not significantly regulated or downregulated e. g. *csgA* (major subunit curli), *csgB* (nucleator), *csgE* and *csgF* (involved in the initiation of curli subunit polymerization) (Römling *et al.* 1998; Keseler *et al.* 2011). Our results represent only the gene expression of loosely associated cells and confirm the results of a study performed by Macarasin *et al* (2012). In their study, curli were shown to be only critical in strong attachment to spinach leaves, whereas loose attachment to spinach was not affected by curli expression. The gene expression of strongly associated cells could not be investigated in our experiment due to technical limitations. Our results differ from the observations of Fink *et al.* who saw stark upregulation of *csgA* and *csgB* on detached lettuce leaves one day after inoculation. The expression levels decreased at day 3 but could be up to 7 times higher in comparison with the control. In their experiments, the pathogens were, however, grown at 37°C (18°C in our study), whereas it is known that curli are especially expressed at colder temperatures. The temperature shift that occurred in the experiment of Fink *et al.* (the lettuce was kept at 25°C) could possibly explain the upregulation of the curli genes. In our experiment no temperature shift occurred and no upregulation could be seen, but we could show that curli were initially expressed as the raw value of e.g. csgA gene was higher than 1000. The same authors, however, found no significant regulation of the generic *E. coli* and *E. coli* O157:H7 *csgAB* genes on lettuce roots although the curli regulator *crl* was induced in generic *E. coli*. But they could demonstrate that the deletion of the *csgA* genes (but not of *csgB*) gene resulted in a reduced capacity of generic *E. coli* to attach to roots (Hou *et al.* 2013). Curli related genes were also expressed during the exponential growth of *E. coli* O157 EDL933 in radish seedlings (Landstorfer *et al.* 2014). It seems that the role of curli is not yet elucidated.

The ability to produce curli seems to be related to the ability of *E. coli* to produce a biofilm (Vidal *et al.* 1998). *bhsA* (formerly known as *ycfR*), biofilm formation through hydrophobicity and stress response, was significantly upregulated in our experiment. Upregulation of *bhsA*, induces repression of biofilm formation by increasing indole synthesis. *bhsA* is upregulated under a variety of stresses such as heavy metals, drastic pH changes, temperature shock, sodium, hydrogen peroxide and, in the presence of sodium salicylate (Pomposiello *et al.* 2001; Zhang *et al.* 2007). Similar to other stress related genes, cells confers resistance against these stresses in response to upregulation of this gene (Zhang *et al.* 2007). Upregulation was also observed by Kyle *et al.* and Fink *et al.* on lettuce leaves and Hou *et al.* on lettuce roots. The last two authors also showed that a deletion mutant showed reduced attachment to both lettuce roots and lettuce leaves.

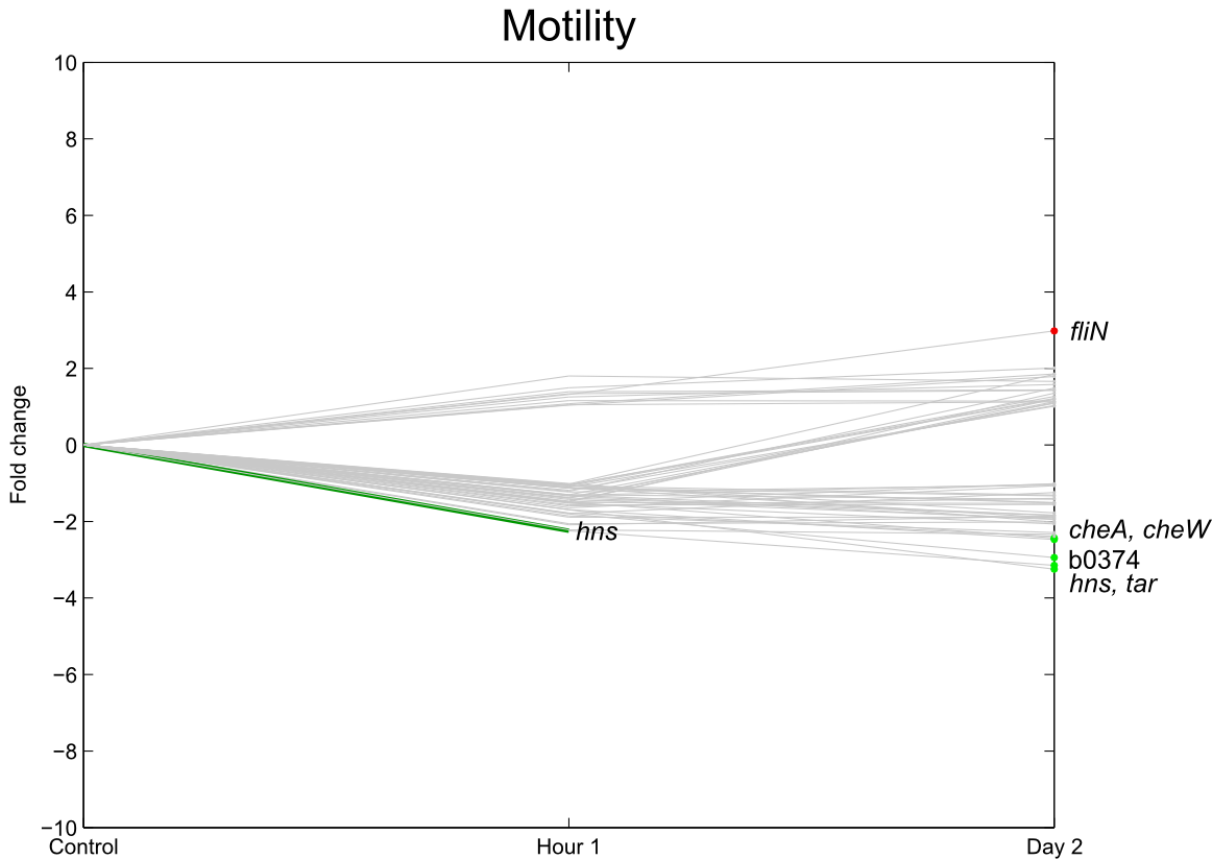


Figure 6.8: Relative expression of *E. coli* O157:H7 Sakai genes related with motility and flagella one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at $0.01 \leq P < 0.05$, dark green: downregulation at $P < 0.01$, light red: upregulation at $0.01 \leq P < 0.05$, dark red: upregulation at $P < 0.01$. Grey: no significant difference in expression. Data represent the mean of four biological replicates.

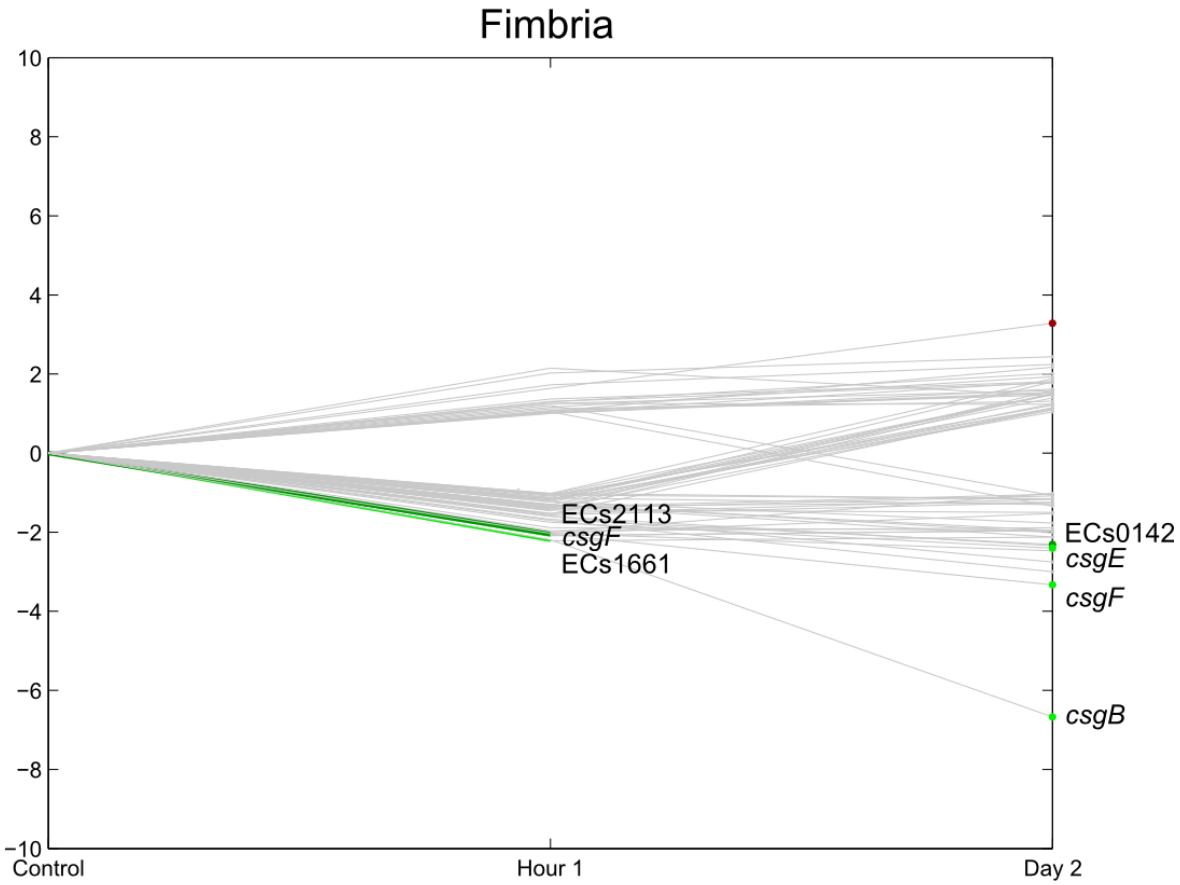


Figure 6.9: Relative expression of *E. coli* O157:H7 Sakai genes related with fimbria one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at $0.01 \leq P < 0.05$, dark green: downregulation at $P < 0.01$, light red: upregulation at $0.01 \leq P < 0.05$, dark red: upregulation at $P < 0.01$. Grey: no significant differences in expression. Data represent the mean of four biological replicates.

6.3.7 *E. coli* O157:H7 Sakai virulence genes

(Figure 6.10). Pathogenic *E. coli* distinguishes itself from generic *E. coli* through the presence of virulence genes. The Sakai O157:H7 chromosome encodes at least 131 proteins that are assumed to have virulence-related functions and that are not present in *E. coli* K12 (Hayashi *et al.* 2001). 31 genes are related to fimbria for which the importance and gene-expression in this study was already discussed above. Other virulence genes are encoding other adhesins/invasin like proteins (at least 14 genes), type III secretions system related proteins and toxin or toxin-like proteins (Hayashi *et al.* 2001).

The type III secretion system encoded by the LEE locus is responsible for the formation of attaching and effacing (A/E) lesion. In *E. coli* O157:H7 Sakai an additional type III secretion system, designated ETT2 (*E. coli* type three secretion 2) was found as well. *ler* (LEE encoded regulator, ECs4588), a central regulator for the genes encoded on the LEE locus was repressed during our experiments (Zhu *et al.*

2006) and consequently most type III secretion system related genes were downregulated, some genes, however, were significantly upregulated, SctW/EivE (ECs3732), ECs4558 (escD) and ECs 4580 (escU).

In general, the majority of virulence genes were downregulated. The best known virulence factors are the Shiga toxins (*stx1* and *stx2*) and to a lesser extent enterohemolysins. On the *E. coli* O157:H7 Sakai genome, a gene encoding for the major virulence factor in *Clostridium difficile* was discovered as well, together with two genes coding for toxin like proteins: ECs0542 (not included on micro array) and ECs1283. ECs1283 was not differentially regulated in our experiment.

Shiga toxin I subunit B precursor (ECS2973) and EHEC-*hlyA* (hemolysin toxin protein) were significantly downregulated two days after inoculation ($P<0.01$). This is in contrast with some post-harvest results from Carey *et al.* who showed that the expression of *stx1* and *stx2* increased on postharvest romaine lettuce at 4°C and 15°C, with higher expression values at 4°C. Also Sharma *et al.* could observe increased expression of virulence genes in post-harvest experimental set-up, but they observed that at 4°C the expression of the virulence genes decreased, whereas at 15°C, an increase in expression could be observed (especially in near ambient conditions compared to modified atmosphere packaging). Also Dino and Bach were able to detect small amounts Shiga toxins in an *E. coli* O157 population which was supposed to be in the VBNC-state after inoculation on lettuce leaves (Dinu and Bach 2011). It should however be noted that the strain that we used had insertional inactivation of the *stx* genes, their expression could, therefore, not accurately be assessed. *eae* was also found to not be differentially regulated. This is in accordance with Xicohtencatl-cortes *et al.* (2009) who showed that the *eae* gene was not involved in attachment to fresh produce. Also Noel *et al.* (2010) found that *Salmonella* motility and animal virulence genes did not contribute significantly to fitness of the bacteria inside tomatoes.

6.3.8 Unknown genes

Almost 40% of the genes that were differentially regulated at $P<0.01$ were poorly characterized or had an unknown function following COG-classification. As most of the research to determine the function of the different *E. coli* genes was done in conditions which simulates the animal (intestine) environment, this may indicate that *E. coli* uses a specific set of genes to survive in the non-animal environment. Interestingly, several of these genes were also Sakai-specific. Seven Sakai-specific genes were significantly upregulated and 20 downregulated at $P<0.01$. By performing a BLAST-search on the nucleotide sequences of these genes, the (putative) function of genes with highly similar or identical sequences could be found in other *E. coli* strains. An overview is given in Table 6.1. A

selection of these genes is discussed in some further detail and are especially interesting for future research.

ECs4115 is possibly similar to *yhcR* which is also known as *aaeX* and seems to be related with biofilm formation. The genes in the AaeXAD operon, which are normally not expressed at a significant level (Tseng *et al.* 1999; Van Dyk *et al.* 2004), were some of the most upregulated genes in a study of global gene expression of biofilms of two *E. coli* urinary tract infection strains grown in human urine (Kvist *et al.* 2008). Furthermore, Monnappa *et al.* (2013) showed that *aaeXAB* may be involved in the efflux of (even low) concentrations of plant hydrolysate-related phenolics, such as ferulic acid, an abundant phenolic phytochemical found in plant cell wall components and vanillic acids (Monnappa *et al.* 2013).

ECs2526: possibly similar to CorC which is a Mg and cobalt efflux protein. It may be possibly that this gene was induced due to the presence of Mg in the $MgSO_4$ buffer.

Ecs0230 possibly RNA methyltransferase type VI secretion protein. The contributions of T6SSs to virulence development are diverse. In cell culture systems T6SSs have been reported to play crucial roles in cell adhesion and invasion and intracellular growth but also interbacterial competition (Kapitein and Mogk 2013).

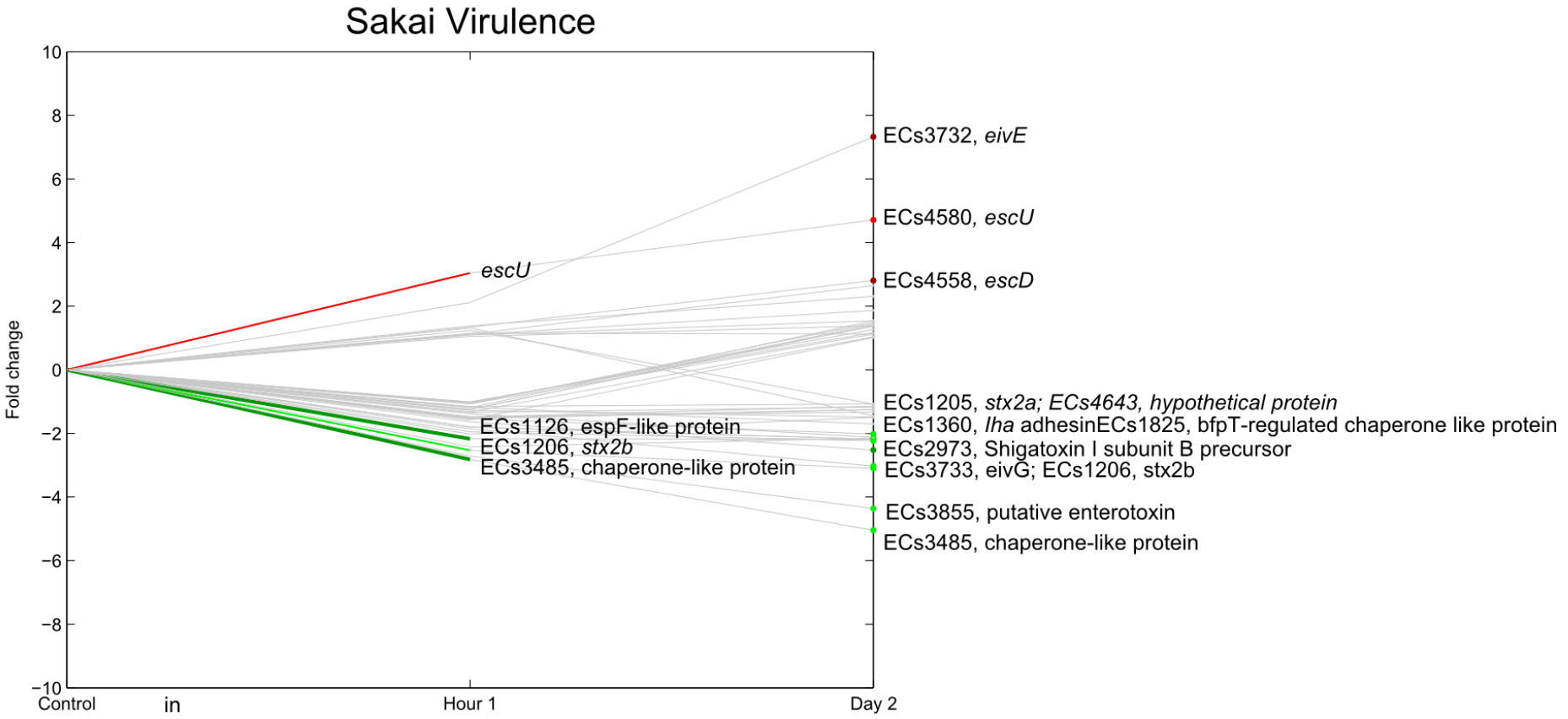


Figure 6.10: Relative expression of *E. coli* O157:H7 Sakai genes related with virulence (except for fimbrial genes) one hour and two days after inoculation on lettuce plants. A colored line between the control and hour 1 shows that a particular gene was significantly differently expressed one hour after inoculation on the lettuce in comparison with the $MgSO_4$ buffer. A colored line between hour 1 and day 2 shows that the gene expression of a particular gene was significantly different between one hour and two days after inoculation. Colored dots at day 2 show the genes which were significantly differently expressed on day 2 in comparison with the control. Light green: down regulation at $0.01 \leq P < 0.05$, dark green: downregulation at $P < 0.01$, light red: upregulation at $0.01 \leq P < 0.05$, dark red: upregulation at $P < 0.01$. Grey: no significant difference in expression. Data represent the mean of four biological replicates.

Table 6.2: Overview of expression levels of genes encoding for hypothetic Sakai specific proteins at P<0.01 level at day 0 or day 2 (significant regulation is shown in bold)

GeneName	Foldchange Day 0	Foldchange Day 2	Similarity with known gene	Strain with known gene with similar sequence	% of similarity
ECs0073	-2.3	**-2.5**	non-LEE-encoded type III secreted effector	*Escherichia* coli O157:H7 str. TW14359	100%
ECs0230	2.0	**3.7**	VCA0109 like protein (RNA methyltransferase type VI secretion protein)	*Escherichia* coli O157:H7 str. EDL933	100%
ECs0238	**-2.4**	-4.4	/	/	/
ECs0281	-1.9	**-2.4**	Putative tail fiber assembly protein	*Escherichia* coli O157:H7 str. EDL933	100%
ECs0541	-2.1	**-2.7**	PKD domain protein	*Escherichia coli* O157:H7 str. EC4115	99%
ECs1125	-1.7	**-2.7**	EspF like protein or tir-cytoskeleton coupling protein	*Escherichia coli* Xuzhou21/ *Escherichia coli* O157:H7 str. TW14359	100% / 100%
ECs1204	1.7	**2.2**	Putative DNA methylase	*Escherichia coli* O157:H7 str. EDL933, complete genome	100%
ECs1238	-2.0	**-3.1**	/	/	/
ECs1239	-1.8	**-2.3**	/	/	/
ECs1246	**-2.2**	-3.7	/	/	/
ECs1445	-1.9	**-3.4**	/	/	/
ECs1567	-2.6	**-3.3**	T3SS effector protein EspO	*Escherichia coli* O145:H28 str. RM12761	100%
ECs1586	-2.9	**-4.0**	/	/	/
ECs2291	-1.6	**-2.2**	Lipoprotein ynfC precursor	*Escherichia coli* O157:H7 str. SS17, complete genome	100%
ECs2473	**-2.0**	-1.5	Putative lipoprotein		
ECs2526	**14.7**	**8.2**	Mg and Co efflux protein CorC	*Escherichia coli* O157:H7 str. EDL933, complete genome	100%
ECs3238	**-3.8**	**-6.2**	/	/	/

GeneName	Foldchange Day 0	Foldchange Day 2	Similarity with known gene	Strain with known gene with similar sequence	% of similarity
ECs3250	-2.1	**-2.2**	56 bp at 5' side: hybrid sensory histidine kinase in regulatory system with EvgA 289 bp at 3' side predicted transporter	a.o. *Escherichia coli* O157:H7 str. EDL933, complete genome	100%
ECs3772	-2.2	**-2.9**	/	/	/
ECs3966	**2.3**	3.5	23S rRNA (guanine-N-2-) - methyltransferase rlmG	a.o. *Escherichia coli* O157:H7 str. EDL933, complete genome	100%
ECs4115	7.6	**25.8**	similar to yhcR which is also known as AaeX	*Escherichia coli* O157:H7 str. EC4115	100%
ECs4465	**-2.6**	**-4.4**	Fic family protein	*Escherichia coli* O157:H7 str. EC4115, complete genome	100%
ECs4491	**3.1**	1.8	Periplasmic septal ring factor with murein hydrolase activity EnvC	*Escherichia coli* O157:H7 str. EDL933, complete genome	100%
ECs4537	**-2.2**	-2.4	transposase ORF B, IS3 family protein	*Escherichia coli* O55:H7 str. RM12579, complete genome	100%
ECs4816	-2.4	**-2.4**	/	/	/
ECs5266	2.0	**2.6**	transposase	*Escherichia coli* O157:H7 str. EDL933, complete genome	100%
ECs5291	-1.9	**-2.9**	adherence and invasion outermembrane protein (Inv,enhances Peyer's patches colonization)	*Escherichia coli* O157:H7 str. EDL933, complete genome	100%

6.4 Conclusions

The main goal of our research was to investigate the gene expression of *E. coli* O157:H7 in a simulated preharvest contamination event in a greenhouse. The adaptation of the pathogen to this new environment was investigated with special interest towards regulation of genes which could have a consequence for the pathogens subsequent survival during processing and consumption. Furthermore, we tried to distinguish whether loosely vs. strongly associated bacteria adapt in a different way to their new environment.

To simulate a preharvest contamination event in a greenhouse, special attention was paid to the culture conditions of the plants and pathogens. Growing, untreated plants were used, grown in the greenhouse. The used pathogen strain, *E. coli* O157:H7 Sakai, was previously related to a fresh produce outbreak. Furthermore, the Sakai strain was grown at 18°C, and suspended in a $MgSO_4$ buffer in order to simulate the suboptimal environmental conditions including unfavorable temperature and nutrient limitation which may occur outside the animal host such as in irrigation water. This is important as it was recently shown by Seo and Matthews (2014) that the medium composition and cultural conditions may have an important influence on the behavior of enteric pathogen on plants. In their experiments, the survival of *E. coli* O157:H7 cells exposed to soil or manure exhibited greater survival on plants compared to LB-grown *E. coli* O157:H7. These researchers support, together with others, the hypothesis that the plant-environment, or more broadly the non-animal environment, is part of the ecological niche of human pathogens (Schikora *et al.* 2008; Shirron and Yaron 2011; Schikora *et al.* 2012a; Schikora *et al.* 2012b; Seo and Matthews 2012; Jayaraman *et al.* 2014). Our results seem to support this hypothesis. Firstly, we were able to show that some genes could have been induced by chemicals produced by the plant defense response, although further research is essential as also other factors may have been responsible for this upregulation. Secondly, taken into consideration that the most important *E. coli* O157:H7 genes that are expressed in an animal environment are known, also the fact that almost 40% of the significantly differentially regulated genes were poorly characterized or had an unknown function support the fact that the pathogen may be genetically adjusted to survive in a non-animal environment. Thirdly, the pathogen relied little on its animal virulence genes, as most virulence genes were downregulated. Quite some overlap in our results could be observed with Fink *et al.*, the most closely related research that could be found, indicating that the adaptation to a plant environment seems to be a robust reaction. In both studies especially stress responsive genes were upregulated whereas energy metabolism and translation mechanisms were downregulated. Some differences could, however, be observed. The most striking difference was the difference in regulation of the curli genes. Different culturing conditions of the pathogens and survival trends on

the lettuce may be responsible for these observations. A standardized culturing and inoculation protocol of the pathogens designed to simulate environmentally stressed cells could avoid such problems in the future. Complementary to our research, studies with mutants (knock-out, overexpression) and challenging tests such as performed by Kyle *et al.* and Oliveira *et al.* should be performed as such experiments may reveal to which extent the observed gene expression changes have an influence on the subsequent survival, antibiotic and sanitation resistance of the pathogen. Kyle *et al.* were already able to shown that pre-exposure to lettuce lysate increased the gene expression of *E. coli* O157 stress genes and effectively increased their resistance against H_2O_2 and induced cross protection against other sanitizers sanitizers (Kyle *et al.* 2010). Oliveira *et al.* did not do gene expression experiments, but they showed that it is not straight forward to link the changes that occur in *Salmonella* Typhimurium when a complete contamination event was simulated to their behavior in the gastric tract (Oliveira *et al.* 2011). They simulated subsequent (cross-) contamination of the soil and lettuce, followed by packing of the lettuce (modified atmosphere) and storage. Consumption of the lettuce was simulated with a simulated gastrointestinal tract system. Although the capability of the pathogens to survive simulated gastric fluid slightly increased, and the capability to grow in the simulated intestinal fluid increased, the capability of epithelial attachment and invasion decreased. As a consequence, the overall survival probability of *S.* Typhimurium in the gastrointestinal tract system was decreased (Oliveira *et al.* 2011).

Unfortunately our results only represents the gene expression of the loosely associated pathogens as we were not able to extract enough RNA from the strongly associated population. The same technical limitations forced us to use high inoculum levels of bacteria, which are much higher than can be expected in a natural contamination event and which are also higher than the natural carrying capacity of lettuce which is around 6.5 log CFU/g (Williams *et al.* 2013). As a consequence, we were not able to observe growth of the pathogen on the leaf. The nutrients seemed not to be enough to sustain the whole population and a decrease of pathogens was observed. Our results should, therefore, be interpreted in this context. Further research investigating the gene expression of enteric pathogens which are proliferating on living plants and of the strongly associated pathogens is challenging due to high amount of RNA that should be obtained, but particularly interesting and to the knowledge of the authors not yet investigated.

Our findings reveal that the pathogen actively interacts with the plant environment by adapting its metabolism and responding to oxidative stress. Consequently, the pathogen may have acquired enhanced resistance against oxidative stress and cross-protection against various other stresses. On the other hand, a decrease in the expression of the Shiga toxin and several other virulence genes was

observed. Further research, is needed to investigate how these adaptations may affect the pathogen's subsequent survival during processing and consumption.

Chapter 7:

General discussion

and future perspectives

7.1 Introduction

The current PhD research was situated within the SALCOSLA project. This project was funded by the Belgian Federal Public Service Health, Food Chain Safety and Environment in the period April 2009 to April 2013 in order to gain more information on the risk factors for introduction and persistence of enteric bacterial pathogens during the Belgian primary production of leafy greens. The request found its origin in the increase in foodborne outbreaks linked to fresh produce in the USA (Aruscavage *et al.* 2006) and in the fact that in Europe some smaller outbreaks occurred (Van Boxstael *et al.* 2012; EFSA 2013). Particular attention was taken to focus the research project towards the Belgian vegetable production situation taking into account Belgian local production conditions and lettuce type. Butterhead lettuce was chosen because of its economic value for Belgium, with production both in open field and greenhouses, and the identified lack of knowledge in peer reviewed literature about this typical northern European lettuce type with regard to routes of contamination and potential for survival of growth of human enteric bacterial pathogens during primary production. Greenhouse butterhead lettuce production was taken as the model production system and *Salmonella* and *E. coli* O157:H7 as model organisms.

A first objective within the SALCOSLA project was to determine the contribution of two potential contamination routes for introduction of *Salmonella* and *E. coli* O157:H7 on the crop during butterhead lettuce production in the greenhouse, namely seeds and irrigation water. Therefore, the survival of four different pathogen strains was studied **i)** on lettuce seeds over a period of two years and **ii)** in five different irrigation water samples over a period of two weeks. The survival of pathogens in manure, organic fertilizers or their derived organic fertilized soils was not studied, as in Belgian greenhouse butterhead lettuce production mainly inorganic fertilizers are used and thus the introduction of enteric pathogens via soil is less likely. A study of Holvoet *et al.* confirmed this and also showed that, if organic fertilizers were used, these were commercially available dry pellets which were microbiological safe (Holvoet 2014).

A second objective of the SALCOSLA project was to gain more insight and knowledge about the behavior of *Salmonella* and *E. coli* O157:H7 on butterhead lettuce during the plant growth cycle. Three different aspects were investigated upon introduction of these enteric bacterial pathogens on the lettuce crop leaves: survival, attachment and gene expression. The survival of the pathogens on butterhead lettuce plants was studied both in the environment of a controlled growth chamber and in an actual setting of a greenhouse. The influence of crop stage in the growth cycle, the leaf's age (or position of the leaves in the crop) and the occurrence of an irrigation treatment were taken into account. Also the effect of prior residence in irrigation water (and thus the exposure to nutrient stress) on the capacity for *E. coli* O157:H7 to attach onto lettuce leaves was investigated. The

attachment of these stressed pathogens was compared with freshly cultured pathogens. Furthermore, to improve the understanding about the underlying genetic mechanisms that the pathogens use during their survival on these foods of non-animal origin (FoNOA), gene expression experiments of *E. coli* O157:H7 were performed on growing butterhead lettuce plants.

For this PhD research, the emphasis was put on laboratory based model studies using artificially inoculated water, seeds or lettuce plants or leaves to study the behavior of pathogens. This enabled to control and standardize the experimental conditions. In addition, the occurrence of natural contaminated samples of butterhead lettuce or its initial seedlings or irrigation waters with enteric pathogens is expected to be very low as shown in the surveillance study for hygiene indicators and enteric pathogens in the Belgian lettuce production as conducted by Holvoet *et al.* (2014). However, in the framework of the current PhD study it was noted that also under conditions of artificial inoculation and standardized controlled conditions, studying the behavior of these human enteric pathogens is challenging as bacterial behavior is variable under stressed conditions. In addition, experiments with growing and respiring plants and leaves or seeds and irrigation water which are variable in both biochemical and microbiological composition, are inherently subjected to variability. In the present discussion, the implications for research regarding human pathogen on plants and more general for food safety are subsequently discussed and the need for standardization of laboratory protocols is addressed.

7.2 Good seed makes a good crop: survival of enteric pathogens on lettuce seeds

In **Chapter 2** it was investigated whether contaminated lettuce seeds could be an introduction route of human enteric pathogens in the lettuce primary production. It was shown that *Salmonella* and *E. coli* O157:H7 were able to survive at least for two years on/in lettuce seeds which were stored in the dark at 18°C. The survival, as determined by conventional plating, of two tested *Salmonella* serovars was significantly better than two tested *E. coli* O157:H7 strains. These observations were in accordance with previously described results on alfalfa seeds (Taormina and Beuchat 1999; Beuchat and Scouten 2002). When the seeds were sown two year after contamination, *Salmonella* as well as *E. coli* O157:H7 were able to proliferate on the seedlings and grew out to high levels (up to 5.92 log CFU/seedling for *Salmonella* inoculated seeds). These results confirmed for *Salmonella*, the findings of Fu *et al.* (2008) who had already shown that *Salmonella* was able to resuscitate on naturally contaminated alfalfa seeds when they were germinated after 8 years. Our findings were especially important in the context of the so called 'EHEC crisis' in Europe in 2011. This outbreak in Germany and France, was most probably linked to the consumption of sprouted fenugreek seeds.

Epidemiological evidence pointed to contaminated seeds as the cause of the outbreak and it could be shown by trace back investigation that the seeds should have been contaminated for at least two years. The implicated seeds from the two outbreaks, originated from a common import of seeds from Egypt two years earlier (EFSA 2011b). It was, however, at the moment of the outbreak, not yet known whether *E. coli* O157:H7 was able to survive for such a long period on seed and subsequently proliferate on the sprouting seeds. So, although it was not the primary goal of the seed experiment, our results proved to be valuable as it was shown that long term survival of pathogenic *E. coli* and its subsequent resuscitation and growth during sprouting of seeds is possible. Furthermore, the results emphasize the importance of the microbiological quality of the seeds during sprouting seed production, and thus also the production of lettuce seedlings commonly used as input for setting up lettuce production.

The main goal of the experiment was, however, to investigate whether seeds should be considered as a contamination source of human enteric pathogens for leafy vegetables. Based on our data, we could show that seeds, if contaminated, will support long-term survival and are thus a potential route for introducing enteric pathogens in the lettuce production process. Furthermore, we showed that detection of the potential pathogen contaminating the seeds can be facilitated by sprouting the seeds. Seeds themselves have a low water activity which may stress the enteric pathogen of concern and may render it hard to get a reliable result of isolation. Prior sprouting of the seeds under conditions of ambient temperature and high humidity will promote, as noted in our experiments, also growth of *Salmonella* and *E. coli* O157:H7 and as such support natural 'enrichment' of potential low pathogen contamination of seeds and thus be a preferred approach in screening seeds for pathogen contamination. This option is also foreseen in EC Regulation nr. 209/2013 regarding the microbiological criteria for foodstuff. The regulation was adjusted after the 'EHEC crisis' occurred and sets requirements for the analysis of seeds to be used for sprouted seeds production: a representative subsample of 0.5% of each seed batch composed of subsamples of 50 g, needs to be germinated for at least 48 h. And five samples of 25 g of this batch need to be analyzed for the presence of *Salmonella* and Shiga toxin producing *E. coli* O157, O26, O111, O103, O145 and O104:H4. Still the detection of pathogens in sprouted seeds is challenging and not fail-safe due to the growth and presence of also high numbers of indigenous microbiota which may interfere with the actual isolation of the enteric pathogens in the sprouted seeds *(*Tzschoppe *et al.* 2012*;* Verstraete *et al.* 2012*).* As an alternative to analysis of seeds or sprouted seeds, the EC Regulation 209/2013 also mentions the option that 5 samples of 200 ml of the spent irrigation water may be analyzed when an appropriate sampling plan is available. As a response on the crisis*,* also requirements regarding registration of sprouting production companies, traceability of the seeds and certification of seeds

for sprouted seeds consumption and requirements of good hygienic production were set ((EU) nr. 852/2004, (EU) nr. 2073/2005, (EU) nr. 209/2013, (EU) nr. 210/2013)). In order to know whether similar requirements should be advisable for seeds of other vegetables or fruits intended to be eaten raw, more information is required about the current microbiological quality of the seeds and the subsequent behavior of the pathogens on the growing plants up to harvest. For this kind of research it is important that inoculation levels similar to natural contamination levels are simulated.

For our experiment, there are some aspects that make it difficult to determine to which extent the use of contaminated seed or seedlings at the set-up of the lettuce crop production cycle actually will also result in the presence of a contaminated butterhead lettuce crop at harvest. Firstly, in our experiment the contaminated seedlings were not further grown to fully mature crops and, therefore, the subsequent survival of the pathogens on the seedling throughout the production cycle which takes up to 12 weeks depending upon the season, is unknown. There is evidence that generic *E. coli* (inoculated at ± log 7 CFU/g seed) could be detected up to 42 days after sowing of freshly contaminated spinach seeds (Warriner *et al.* 2003a). But on the other hand it was already shown that occasional pathogen contamination on leafy greens dies-off during the course of growth of the lettuce plant, although this may differ depending upon climatic conditions (Aruscavage *et al.* 2008; Zhang *et al.* 2009; Wood *et al.* 2010). Furthermore, it was shown that some indigenous bacteria on the plant may show antagonistic activity towards enteric human pathogens and reduce the attachment capacity of the pathogens. On the other hand, the indigenous bacteria may also prolong the survival of the enteric pathogens on the plants by e. g modifying the tissue (for example by increasing the nutrient production of the plant) and supplying a suitable niche (for example by producing an polysaccharide matrix) (Cooley *et al.* 2003; Cooley *et al.* 2006; Lima *et al.* 2013). Moreover, it needs to be noted that untreated seeds such as used in our experiments are typically not used during commercial lettuce production. Instead, fungicide treated coated seeds are used. The influence of this fungicide coating may have an impact on the survival of the pathogens on the seeds, but is not yet known.

Last, currently there is little information available about the overall microbial quality or the presence of enteric pathogens such as *Salmonella* or *E. coli* O157 in lettuce seeds or any other seeds intended for vegetable and fresh fruit production. In Belgium, seed producers are only obliged to test the microbiological quality of the seeds when they want to export them to countries with specific phytosanitary requirements (Federaal Agenschap voor Veiligheid van de Voedselketen (FAVV) 2013) . The most actual information about the food safety of butterhead lettuce seedling for Belgium was obtained by Holvoet *et al.* In their study lettuce seedlings and seedling peat-soil were tested for the presence of human pathogens (*stx* genes, *Salmonella*, *Campylobacter*) but they did not detect any

positive results in any of the 80 samples tested, although occasionally some generic *E. coli* could be enumerated.

7.3 In deep water? Survival of enteric pathogens in irrigation water

To test to which extent human enteric pathogens may persist in irrigation water and thus serve as a likely route of introduction of pathogens to the lettuce crop , the survival of 4 pathogenic strains (2 *E. coli* O157:H7 and 2 *Salmonella* serovars) was monitored in samples taken from the water reservoirs or groundwater being used for irrigation at four Belgian butterhead lettuce production sites and the ILVO greenhouse (**Chapter 4**). It was shown that during the 2 weeks storage period both temperature and microbiological and chemical water quality had an influence on the survival characteristics of the pathogens in the irrigation waters. Cold temperature (4°C) enhanced the survival of the pathogens in this nutrient poor water, whereas at higher water temperatures (20°C), the natural background microbiota and the pathogens were probably more metabolically active so that competition could be observed, with less survival for the enteric pathogens. By removing the background microbiota and by using lower inoculum levels, we could show that also the chemical composition of the different irrigation water samples which were highly different (in particular high Zn levels in one of the water samples) could have had an important impact on the survival of the pathogens at 20°C. Still a substantial variation in the survival characteristics was observed: in some irrigation water samples a decrease of the pathogens could be seen, whereas, in other samples growth of the pathogen could be observed. During the experiment only a restricted number of microbiological parameters were determined using standard cultural methods, further investigation of the microbiota using also non-culture based methods as well as a number of biochemical analyses of the irrigation waters is warranted in future investigations.

Based on these results which point to a longer persistence of pathogens at colder temperatures, one could hypothesize that a prolonged risk for transfer of enteric pathogens from irrigation water to the lettuce crop would occur during colder seasons of the year. Still, the actual occurrence of these human enteric pathogens in the irrigation water may be variable and depends upon the actual environmental pressures (e.g. the close proximity of livestock which may carry enteric pathogens, pastures or wild animal activities (including birds, rodents) (FAO 2003; Brandl 2006; BioHaz 2014) and climatic conditions e.g. heavy rainfall may promote introduction of fecal contaminated run-off water from nearby fields (Liu *et al.* 2013). In a recent study from Holvoet *et al.* it was demonstrated that the presence of pathogens in irrigation water samples from Belgian lettuce production farms showed a seasonal pattern with more pathogens being present in the water during months with higher temperature (Holvoet *et al.* 2014). So, despite a lower survival of pathogens in irrigation water in the

warmer season, the likelihood of introducing pathogens into the irrigation water, in particular surface water, seems to be higher during this period.

In the same study from Holvoet *et al.* it was demonstrated that enteric pathogens may effectively be present in Belgian irrigation water used by lettuce producers (both greenhouse and open field). Twenty-five percent of all water samples (30/120) showed to be positive for either *Salmonella* spp., *Campylobacter* spp. or EHEC (*stx* and *eae* genes detected by PCR) during the whole study (1.5 year). Mainly *Campylobacter* was found (35/120). Combined presence of *stx* and *eae* genes were detected by PCR in 6/120 samples, but a pathogenic *E. coli* strain could only be isolated from two samples. *Salmonella* was isolated from one irrigation water sample. Especially open well water (21/51 samples positive for at least one of the pathogens) was identified as a risk factor, whereas, groundwater was found to be less contaminated (3/17 samples).

In practice, most of the greenhouse lettuce producers have, in contrast to the open field growers, implemented a water treatment system which is used to prevent production loss due to plant diseases transmitted by irrigation water such as bacterial midrib rot caused by *Pseudomonas chicorii*. The common systems are disinfection through chlorination, hydrogen peroxide or UV-treatment, also prior sand filtration is sometimes used. The disinfection system is mostly activated the whole year round, although some producers only use it during autumn when the risk for bacterial midrib rot is the highest (Cottyn *et al.* 2009; Holvoet 2014). Holvoet *et al.* found that when disinfection of irrigation water was used, no pathogens were observed in the water sampled at the tap in contrast to the water samples at the source (Holvoet 2014). A risk factor for greenhouse lettuce production seems, therefore, to be the fact that the water disinfection installation is not always continuously used by some lettuce growers. During the period that the water disinfection treatment is not used, which is mainly in summer, higher prevalence of pathogens was recorded (Holvoet 2014). During this period, pathogens may contaminate produce but may be established inside biofilm in the sprinkler tubes as well, causing an potentially dangerous situation of persistent contamination. The persistence and even growth of generic *E. coli* in aluminum sprinkler tubes was already established (Pachepsky *et al.* 2012), although no information could be found about human enteric pathogens.

Results from Holvoet *et al.* indicated that human pathogens may actually be present in the irrigation water samples, in particular when using surface water or collected rainfall water in reservoirs which are vulnerable to ingress of fecal contaminated run-off water. In addition, from results in the present PhD study it could be shown that the pathogens' capacity to survive in the irrigation water samples was also highly variable. It seems that the extent to which the occasionally introduced enteric pathogen can actually persist and be transferred to the crop when using the irrigation water is also

dependent on the specific water composition. Consequently, the presence of human enteric pathogens will depend upon the specific lettuce production location, construction of its water system and water quality.

7.4 A bugs life: insights in the behavior of enteric pathogens on plants

The survival of enteric pathogens (*Salmonella* and *E. coli* O157:H7) during butterhead lettuce production was investigated on young and nearly mature butterhead lettuce plants and the effect of overhead irrigation was taken into account (**Chapter3**). Our results showed that when enteric pathogens were artificially introduced on butterhead lettuce plants at a level of 3-4 log CFU/g leaf, they were able to survive on the plant leaves. In the growth chamber, growth up to 2 log CFU/g of the pathogen could be observed and the pathogen level remained stable between day 4 and day 8 after inoculation. On the contrary, the pathogen's survival on lettuce growing in the greenhouse was significantly lower. Growth could on average not be observed and the pathogen concentration continuously declined, leading to the pathogen no longer being detected in some samples after 8 days after inoculation (3/54). This decline was to be expected given the higher variation in relative humidity and temperature in the greenhouse as opposed to controlled and stable growth chamber conditions.

Pathogen proliferation was more clear during the growth chamber experiments, although it could be sporadically observed in the greenhouse as well on individual leaves. Furthermore, the survival of the pathogen was dependent of the leaf age. On young plants, greater proliferation of both *Salmonella* and *E. coli* O157 could be found on middle and old leaves, whereas on nearly mature plants, better survival was observed on young (inner) and old (outer) leaves. Probably not only the nutrient availability on the leaves determined the survival but also microclimate (e.g. susceptibility for dehydration) determined by the position of the leaf in the crop. Eight days after inoculation, the leaf age effect (and/or effect of the position of the leaf in the crop) could not be observed anymore. Leaf age dependent survival was first demonstrated by Brandl and Amundson (2008) under conditions of high relative humidity. Our results confirmed the findings of Brandl and Amundson (2008) but also showed that this effect can be surpassed by environmental factors, introducing high variability in pathogen survival. Our observations may be useful for the design of a risk based sampling plan. For monitoring the hygienic conditions during the whole production cycle, analyzing the outer leaves of the crops seems to be interesting. The pathogens survived quite well on these leaves and outer leaves received irrigation water during almost the whole production cycle and they make contact with the soil. After harvest, sometimes the outer leaves are removed before the lettuce is sold to the

auction or wholesaler. For a food safety authorities, inner leaves seems to be more suitable to be analyzed as they seem to have the ability to carry the highest pathogen levels and are the soft and folded leaves which are preferably consumed.

Furthermore, we were able to show that overhead irrigation could increase the survival of the pathogens in some situations. Increased pathogen survival after overhead irrigation could only be observed a few days after the contamination event, on the inner leaves of nearly mature crops in the greenhouse. We hypothesize that overhead irrigation could have increased the relative humidity and leaf wetness locally in the greenhouse plants, creating more suitable conditions for pathogen survival. It was already shown that enteric pathogens present on plants growing at a low relative humidity may resuscitate or start to proliferate again after exposure to high relative humidity (Brandl and Mandrell 2002). Our findings are important as in Belgium the majority of the lettuce producers and all producers of our study apply overhead irrigation with a sprinkler system (Holvoet 2014). Overhead irrigation is generally considered to be related with a higher risk for contamination as the pathogen comes directly in contact with the edible part of the plants (Gil *et al.* 2013). To lower the risk of pathogen transfer, subsurface or drip irrigation which results in no wetting of the edible portions of the plants can be applied (BioHaz 2014). In the growth chamber with a constant high relative humidity, overhead irrigation did not influence the survival of *Salmonella* or *E. coli* O157:H7 on young or nearly mature plants, and the survival was in general better than in the greenhouse experiment. This confirms that pathogen survival is more likely in periods with high relative humidity.

In the greenhouse, the environmental conditions are less controlled and more variable results were observed which emphasized the difficulty to extrapolate some research findings to actual conditions in practice. Follow-up field or greenhouse studies are recommended to make more 'definite' conclusions and should preferably be performed over different crop production cycles, different seasons and if possible different years and with different lettuce varieties. To simulate a natural contamination event even better, lettuce could be irrigated with pathogens suspended in different types of irrigation water. In fact, our result from the irrigation water study (**Chapter 4**) shows that the irrigation water quality could have an influence on the survival of the pathogen as well. The latter experiments were performed on detached lettuce leaves, stored under high relative humidity. No information is, however, available for a production situation. Furthermore, other factors may influence the pathogen's behavior as well and could be considered for future experiments e.g. the influence of the initial natural microbiota. Indeed, recently a negative correlation could be found between the initial natural microbiota and the survival capacity of *E. coli* O157:H7 (Williams *et al.* 2013). Furthermore, evidence was given that the presence of certain bacterial species may reduce (e.g. *Erwinia*) or enhance the risk (e.g. phytopathogens such as *Dickeya dadantii*) (Goudeau *et al.*

2013; Williams *et al.* 2013). Also pesticide treatments could be taken into account, it was already shown for some pesticide treatments during lettuce production that pathogen survival decreased (Guan *et al.* 2005), whereas, other researchers found that foliar contact application may elevate the risk during tomato production (Lopez-Velasco *et al.* 2013). This type of research will require a separate research project.

7.5 A dual lifestyle: gut versus environment or how to cope with stress

During the different experiments regarding survival on lettuce seeds, lettuce plants and in irrigation water it became clear that there is only a thin line between growth, survival and inactivation of the pathogen: the sprouting process of seeds created good conditions for the pathogens to start actively growing again on the sprouts after two years of decline on the seeds; removing the background microbiota from a water sample could make the difference between pathogen decline or growth; and the position of the leaf in the crop on which the pathogen attached determined as well whether the pathogen was going to be able to proliferate or not.

Environmental conditions are in general considered stressful as nutrient deprivation, physical stresses such as fluctuations in relative humidity, temperature and chemical stresses (pH, reactive oxygen species) are likely to occur. In order to survive such hostile environments, bacteria developed survival strategies. These strategies can be basically classified as 'adaptation' and 'avoidance' strategies (Dinu *et al.* 2009). The 'adaptation' strategy is based on genetic and/or physiological adaptation of the pathogens to enhance tolerance against the stress. The adaptation response that is best known is the individual genetic and phenotypic adaptation of bacteria which may be a stress specific response or a general response. A specific bacterial stress response enables bacteria to protect vital processes, repair damage and restore cellular homeostasis. This response can be evoked by different stresses (acid, heat, cold, etc.) and is dependent on the specific stress, although overlapping responses are described. A more global stress response adaptation, triggered by the alternative sigma factor Rpos may, on the other hand, provide protection from many types of stress (Aertsen and Michiels 2004; Dinu *et al.* 2009). Recently, also the induction in the viable but non culturable state (VBNC) and the induction of phenotypic heterogeneity and genetic diversity are considered as adaptation strategies to stress (Aertsen and Michiels 2004). A second strategy that the pathogens have developed is avoiding the stress. Formation of aggregates in protective niches, localization in biofilms, and internalization in plants were already described, but were not specifically investigated during our studies (Dinu *et al.* 2009).

A specific experiment, testing the influence of pathogen stress on the pathogen's attachment to leaves was conducted in **Chapter 5**. A situation in which the pathogens were adapted to stress by prior residence in irrigation water at 4°C or 20°C for up to 6 days before getting in contact with lettuce leaves was simulated in a lab scale experiment. Attachment to lettuce leaves did not seem to be substantially influenced by temperature or residence time in the water. Also during the gene expression experiments (**Chapter 6**) it was noted that attachment-related *E. coli* O157:H7 Sakai genes were not significantly upregulated when the pathogen was present on growing butterhead lettuce.

Other stress responses could, however, be clearly observed during the gene expression experiment in **Chapter 6** which investigated the expression of all *E. coli* O157:H7 Sakai genes after 1 hour and 2 days residence on young growing butterhead lettuce plants (in the growth chamber at 18°C and 75% RH). Plate counts revealed that on the plant, the pathogen was in a survival mode rather than in a growth permissive state, an indication of stress. Whereas the gene expression data showed increased expression of stress responsive genes: mainly Fe-S cluster related genes and the *marRAB* operon. Fe-S clusters participate in many vital cellular functions such as DNA repair, transcriptional regulation, nucleotide and amino acid biosynthesis and energy metabolism (Schwartz *et al.* 2000). The *marRAB* operon, on the other hand, is involved in multiple mechanisms that bacteria possess to survive exposure to various chemical stresses and antimicrobial compounds such as antibiotics (Fontecave *et al.* 2005; Wang *et al.* 2009). Whatever the underlying cause for this induction of the stress genes (e.g. nutrient scarcity, presence of reactive oxygen species or reaction to salicylates produced by the plant immunity response), upregulation of these genes is linked with enhanced resistance against other stresses such as antibiotics and sanitizers (Pomposiello *et al.* 2001; Fontecave *et al.* 2005; Wang *et al.* 2009; Kyle *et al.* 2010).

Up to now, only limited attention is given to the induction of stress adaptation of human enteric pathogens on plants. Nevertheless, this can have important implications for the subsequent behavior of the pathogens throughout the whole production chain. As our gene expression results especially describe the gene expression modifications that occur in the loosely associated pathogens when die off occurred, this can be interesting for post-harvest research as these are the bacteria that are removed after washing of the lettuce, and are subjected to sanitizers in the wash water. Follow-up tests should, therefore, reveal which implications these gene expression findings would have in practical situations. Can increased resistance be observed to sanitation agents that might be applied in washing baths to avoid cross contamination in a post-harvest settings in the fresh-cut industry? Does *Salmonella* react in the same way? If increased resistance against disinfectant agents occurs due to adaptive response, the current sanitation experiments reported in peer reviewed literature

probably underestimate the effectiveness of the sanitizers as the experiments are usually performed with 'freshly' inoculated cultures on post-harvest lettuce.

Induction of cells in the viable but non culturable state is considered as another 'adaptation' strategy in order to cope with environmental stress. It can be seen as a state of low metabolic activity in which the cells do not undergo cellular division, and as a consequence they are not able to grow on (non-selective) culturing media such as TSA anymore. VBNC is the subject of intense debate and different theories exists for its occurrence. Some investigators see it as a phase before die off. A special altruistic hypothesis of this die off theory is the one where cells in the VBNC release organic molecules that can be used by the other cells to repair their cell membranes and other damage, whereas the VBNC cells die. Others believe that the VBNC cells become again culturable under more convenient environmental conditions. Following the scouting theory, VBNC cells wake up from time to time to test whether the environmental conditions are already more suitable and subsequently wake up the other cells by molecular signaling (Oliver 2005; Barcina and Arana 2009; Dinu *et al.* 2009; Epstein 2009). Although the present PhD research was not specifically focused on it, there were some indications for the induction of cells into the VBNC state and stronger evidence for the formation of sublethally damaged cells on/in seeds, water and lettuce plants. Sublethally damaged cells are different from VBNC as they are defined to be able to grow on non-selective medium but not on commonly used selective media (XLD or CT-SMAC in our case) (Wu 2008). In general our methodology was not specifically adjusted to be able to differentiate between the two statuses. On lettuce seeds (**Chapter 2**) we were able to show that the amount of *E. coli* O157:H7 detected on the lettuce seeds after two years was significantly lower than could be expected based on the percentage of contaminated seedlings. Samples of 200 seeds from 2-year old inoculated seed batches needed to be enriched for detection (<1.3 log CFU/g seed) whereas 12.5 %of the seeds (and thus seedlings) still seemed to be contaminated. Taking into consideration that 1 g of lettuce seeds contained approximately 1000 seeds, the samples of 200 seeds should not have been enriched if the pathogens were able to grow on the selective medium. Also in irrigation water, significant differences in counting results could be seen in some water samples (e.g. up to almost 4 log CFU/ml in groundwater sample 3 for *E. coli* O157:H7) between selective and non-selective medium (**Chapter 3**), whereas live/dead qPCR during the attachment experiment showed that after 6 days, a part of the population could not be detected with plate culturing technique (selective medium with a resuscitation top layer) and pointed towards the presence of VBNC cells (**Chapter 5**). At the second day of the gene expression experiments, we found a higher total RNA content in comparison with what could be expected from the plate counts on selective medium (**Chapter 6**). Plate counting on

non-selective plates were however not done, so we could not distinguish between VBNC and sublethally injured cells.

The presence of VBNC and the induction of the VBNC in foods and especially in fresh produce is already described (Dreux *et al.* 2007b; Dinu *et al.* 2009; Dinu and Bach 2011) and a slight indication for its presence was found in the present study. It was also shown that VBNC cells of pathogenic *E. coli* on lettuce were still able to produce Shiga toxins (Dinu and Bach 2011). From our gene-expression results, however, we noted a down regulation in expression of Shiga toxin genes during the pathogen's decline on the leaf, although this does not necessarily means that Shiga toxins could not be produced anymore. The presence of VBNC human enteric pathogens in the fresh produce chain, may lead to underestimation of pathogenic bacteria and it is still unclear whether the cells may become infective again. The fact that for the detection of VBNC cells mainly molecular or microscopical methods are applied using other basic principles of detection than culture methods makes the detection and interpretation of findings difficult (Elizaquível *et al.* 2014). A first method of detection is based on the detection of metabolically active cells: mRNA is extracted from the sample, cDNA is made for genes of interest and a (quantitative) PCR is conducted. The instability and degradation of RNA is, however, still an issue, furthermore, the same problems as with other PCR methods occur as discussed below. The other methods are mainly based on the fact that dead cells are considered to be membrane compromised and the fact that some dyes may penetrate the intact membrane and others not. A drawback of this technique is that not all dead cells are membrane compromised e.g. UV-killed cells. Microscopy or flow cytometry methods do use this principle but do not provide sufficient specificity or sensitivity to detect specific target organisms as needed in the current PhD study. Also quantitative PCR techniques with sample treatment with DNA-binding molecules such as propidium monoazide (PMA) or ethidium monoazide (EMA) detect only cells with intact membranes. The PMA-method was used in **Chapter 5**. PCR techniques have also some limitations regarding the tested matrix (e.g. inhibitory components) which need to be optimized but the main drawback inherent to PCR-based methods is the high limit of quantification: 1 cell per PCR reaction corresponds to ca. 1000 cells per g (Elizaquível *et al.* 2014). To find out the actual importance of VBNC human enteric pathogens in the fresh produce chain, further research regarding the optimization of the detection of VBNC, detailed knowledge of the prevalence of the VBNC-state, and the food safety risk associated with the VBNC response is needed.

7.6 The need for standardization

From an historical point of view, mainly two research disciplines started working on research regarding human enteric pathogens on plants: i) food safety experts, originally focusing on foods of

animal origin were drawn to study foods of non-animal origin and their routes of contamination at plant primary production as these type of foods were increasingly implicated in food borne outbreaks; ii) plant pathologists or plant physiologists whose initial focus lies on the behavior of plant pathogens on crops and who were studying plant-pathogen interactions also expanded their expertise to human enteric pathogens. Furthermore, also other disciplines such as water microbiologists and soil microbiologists and to a lesser extent veterinary epidemiologists recently show interest in this new discipline as this research of food safety of ready-to-eat products of non-animal origin, freshly eaten as salads, needs a farm to fork approach.

During the present PhD research a wide variety in research protocols was found during screening the peer reviewed literature (see Figure 7.1), making it difficult to decide which protocols needed to be preferred to mimic a natural contamination event in the lab which was at the same time suitable to control experimental conditions in order to establish statistical exploitation of results and relevant findings. Before starting an experiment, the experimental conditions need to be critically evaluated and a lot of decisions need to be taken regarding the type and growth stage and cultivation system of vegetables to be used, the option of taking detached leaves versus growing crops, the selected bacterial strain and prior growth conditions, the environmental factors to be varied or not, the detection methods fit for purpose, the number of replicate samples and sampling frequency etc. It was already shown that small differences in e.g. inoculation conditions may have substantial influence on the subsequent results. *E. coli* O157:H7 survival at 5°C on cut lettuce was significantly better for cultures grown at 15 or 37°C in minimal medium and to late stationary phase in comparison with different growth phases cultured in TSB (Theofel and Harris 2009).

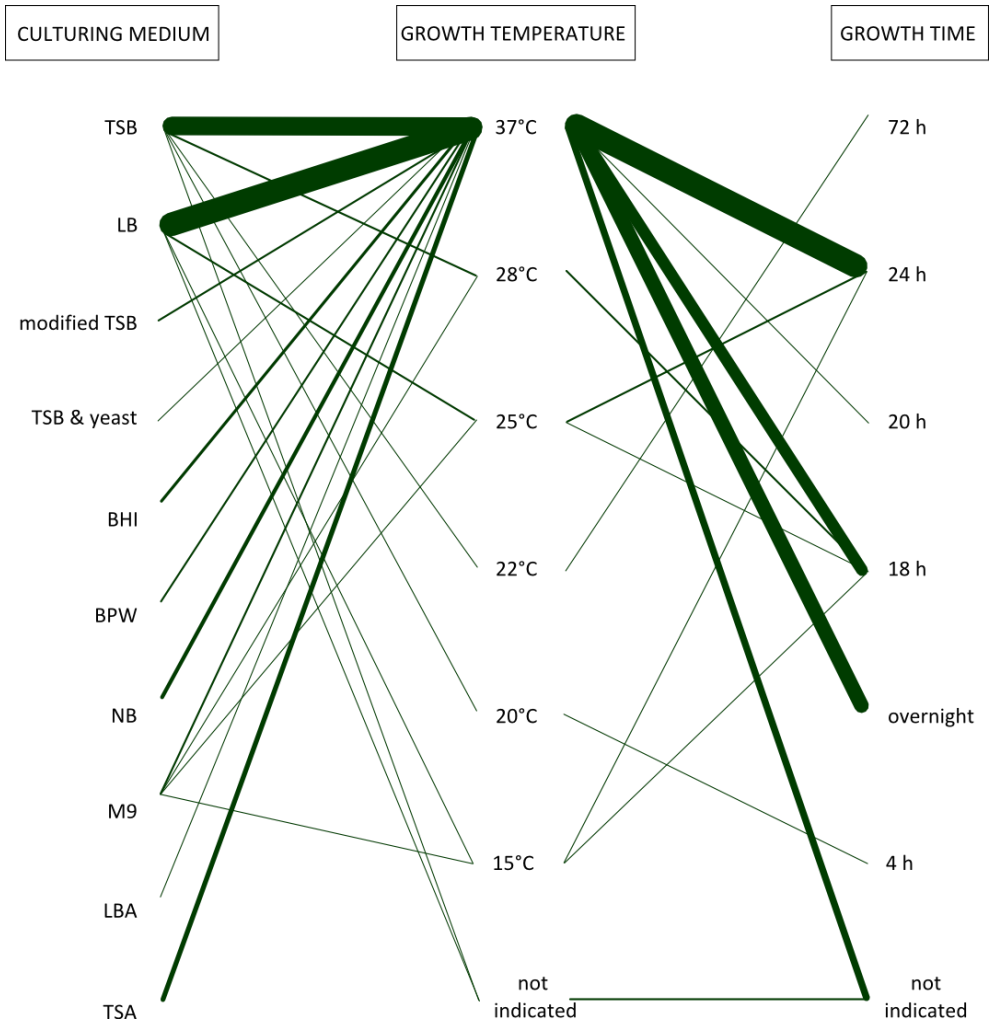


Figure 7.1: Culturing conditions used in 65 articles regarding preharvest contamination of lettuce with *E. coli* O157:H7 and/or *E. coli*.

Salmonella displayed higher affinity toward the abaxial side (up) compared to the adaxial side of Romain lettuce (Kroupitski *et al.* 2011), also *Salmonella*'s survival is considered to be better on the abaxial side in comparison with the adaxial side (Zhang *et al.* 2009), sanitizer's efficiency was shown to be dependent on the inoculation method (dip, spot, spray) with higher efficiency on drop inoculated lettuce leaves (Singh *et al.* 2002). Also for gene expression studies, small differences in experimental set-up can induce substantial differences in the results. This type of study typically tests a small number of experimental conditions and generate a large number of results (regulation of thousands of genes). Each difference in experimental condition may influence the expression of tens to hundreds of genes and overlapping reactions may occur. For the gene expression study of Fink *et al.* (2012), which is most closely related to our study, 8 differences in experimental set-up could easily be found: growth medium, growth temperature, control condition, *E. coli* O157:H7 strain, lettuce type, lettuce incubation conditions, tested time points, treatment of the leaves (disinfection).

As research is advancing, the time is ripe to perform more interdisciplinary experiments such as proposed earlier in this discussion: e.g. combining pre- and postharvest set-ups and thus a continuous flow of monitoring of pathogens on crops starting from primary production over harvest, storage and post-harvest treatments which mimic the commercial production situation as closely as possible (Oliveira *et al.* 2011); research with regard to the behavior of stressed pathogens on plants but also investigating the interaction between the plant and the pathogen at the same time (e.g. regarding physiological plant defense response) which is to the knowledge of the author not yet performed; or research with regard to the interaction between the background microbiota and the pathogens by e.g. non-cultivation dependent techniques such as metagenomics (Lopez-Velasco *et al.* 2010; Lopez-Velasco *et al.* 2011; Lopez-Velasco *et al.* 2012). These kind of experiments combine, however, knowledge of different research fields and are consequently even more complex regarding experimental set-up. The use of standardized protocols may facilitate choices for experimental set-up, increase consistency among studies and enable direct comparison or at least facilitate meta-analysis.

A few initiatives were already undertaken to offer a guideline for experimental design related to human enteric pathogen on plants. In 2001 a standardization protocol was proposed for testing the efficiency of sanitizers in a postharvest set up (Beuchat *et al.* 2001). Also Sinclair *et al.* (2012) attributed to standardization by describing criteria to select surrogate strains. In 2012 and 2013, frameworks were published for designing research studies that would investigate the preharvest microbial food safety hazards and control measures of fresh produce related to contamination due to contact with agricultural water or soil (Harris *et al.* 2012; Harris *et al.* 2013). These two publications contained a.o. a list with good, better and best practices, a list with attenuated an nonpathogenic surrogates that have already been used in model studies or field based studies and a check list for minimal information that need to be provided when publishing. These guidelines offer a lot of information and are especially valuable for researchers that are starting to investigate this topic. The guidelines are specifically focused on applied research but still offer a lot of choices e.g. regarding the inoculum preparation (temperature, medium type, statically incubation or not, suspension medium, etc.). This can be backed up by the fact that each particular contamination event has its own characteristics which need to be mimicked as good as possible.

For more fundamental research investigating basic mechanisms such as pathogen attachment, biofilm formation, internalization etc. no such guidelines are available. For this particular research a model system which describes a confined set of research criteria seems to be an essential prerequisite in the opinion of the author. In such an ideal system a well described reference strain for different pathogens of interest (e.g. a strain for which the whole genome sequence is known and for

which well tested mutants exists) and a well-known model plant species should be chosen. Furthermore, conditions regarding bacterial cultivation, method of inoculation, temperature regime etc. should be well defined (Seo and Matthews 2014). The proposed model system, should however not compromise other research investigating the behavior of other strains, agricultural practices, plant varieties etc. By investigating the conditions of interest and incorporating a treatment that fulfills the requirements of the model system, scientific value is added as comparison with other research studies becomes easier.

Additionally, a research database (preferably open access) which enable to search for specific methodology should be useful as well in order to be able to compare different studies more easily. Such a database will also enable researchers to define research gaps more easily, similar to what was done in the general introduction in this study. A system such as proposed exists already for classical phytopatology research at European level and is implemented by the European and Mediterranean plant protection organization (EPPO, www.eppo.int). EPPO is an intergovernmental organization responsible for European cooperation in plant health. Its objectives are to protect plants, to develop international strategies against the introduction and spread of dangerous pests and to promote safe and effective control methods. Therefore, a large number of standards and publications on plant pests, phytosanitary regulations, and plant protection products were produced and made available on its website. General standards are available in different languages (English, French, sometimes Russian) and specific standard can be obtained by EPPO members. Furthermore, the organization provides databases with detailed information on the geographical distribution and host plants of quarantine pests, online database which provides preferred scientific names, synonyms, common names, EPPO codes, and taxonomic relationships of organisms important in agriculture and crop protection, online database on diagnostic expertise. Experts from more than 100 diagnostic laboratories of the EPPO region have provided details about the pests they can diagnose and the methods they use. Furthermore, they developed software for pest risk analysis (EPPO 2014).

An organization which could have a similar role as EPPO could be the recently proposed research coordination network regarding human pathogens on plants (HPOP RCN) (Fletcher *et al.* 2013). The research coordination network was proposed to be involved in defining research priorities, collaborations between government, education, economy (agriculture and industry) and science (plant pathology, food microbiology and epidemiology). It could, in the opinion of the author, be eminently suitable for the development of a model system or comparative research database as well.

Summary

Fruits and vegetables, together called fresh produce, represent an important part of the human diet. However, since the mid 1990's foodborne illness outbreaks linked to consumption of fresh fruits and vegetables were documented. Especially leafy vegetables, such as lettuce, are frequently involved. Most identified outbreaks were caused by *Salmonella* and *Escherichia coli* O157:H7 (*E. coli* O157:H7), enteric bacterial pathogens which are traditionally associated with food products of animal origin like meat, dairy and eggs. Although the problem especially occurs in the USA, also in Europe outbreaks occurred. Only recently in 2011, over the course of the project, one of the biggest outbreaks occurred in Germany and France. This outbreak, also called 'EHEC crisis' could probably be traced back to the consumption of fenugreek sprouts contaminated with *E. coli* O104:H4, a less common pathogenic *E. coli* serovar. The very severe consequences for public health and significant economic impact underscored the importance to gain more information on the risk factors for introduction and persistence of *E. coli* O157:H7 and *Salmonella* during the primary production of fresh produce.

For this thesis, butterhead lettuce was chosen as model plant because of its economic value for Belgium and the lack of knowledge about this typical northern European lettuce type regarding contamination with bacterial enteric pathogens. The thesis focused on contamination of the crop before harvest, also called preharvest contamination, by using artificial contaminations of seeds, irrigation water and growing lettuce plants. Furthermore, to better understand a natural contamination event, not only the behavior of freshly cultured, unstressed pathogens but also the behavior of stressed pathogens was investigated.

A first objective was to determine the contribution of two potential contamination routes for introduction of *Salmonella* and *E. coli* O157:H7 on the crop during butterhead lettuce production in the greenhouse, namely seeds and irrigation water. Therefore, the survival of four different pathogen strains was studied **i)** on lettuce seeds over a period of two years and **ii)** in five different irrigation water samples over a period of two weeks. Our results confirmed that both seeds and irrigation water may be a potential source of contamination. *E. coli* O157:H7 and *Salmonella* were able to survive for more than two years on lettuce seeds but also to proliferate on the lettuce seedlings even after residing for a period of two years on the lettuce seeds. *Salmonella* survived significantly better on the seeds in comparison with *E. coli* O157:H7. The pathogens were also able to survive in irrigation water, their survival capacity was strain-dependent and varied with storage-temperature of the water (better pathogen survival at 4°C in comparison with at 20°C). But most importantly, our results showed that irrigation water samples from different Belgian lettuce growers which showed a high variability in chemical and bacteriological composition, may have a different capacity to transmit enteric pathogens to the crops because of a different survival profile of the pathogen in each water sample.

A second objective was to gain more insight and knowledge about the behavior of *Salmonella* and *E. coli* O157:H7 on butterhead lettuce during the plant growth cycle. Three different aspects were investigated upon introduction of these enteric bacterial pathogens on the lettuce crop leaves: survival, attachment and gene expression.

The survival of the pathogens on butterhead lettuce plants was studied both in the environment of a controlled growth chamber and in an actual setting of a greenhouse. The influence of crop stage in the growth cycle, the leaf's age (or position of the leaves in the crop) and the occurrence of an irrigation treatment were taken into account. The survival of both pathogens (*Salmonella* and *E. coli* O157:H7) was investigated on young and mature butterhead lettuce plants and the effect of overhead irrigation was taken into account. Our results showed that when enteric pathogens were introduced on butterhead lettuce plants, they were able to survive on the plant leaves. Bacterial survival and growth rate in association with the lettuce leaves was highly variable and this variation was dependent on the crop growth stage and strongly dependent on the environmental conditions, especially the relative humidity. The pathogen's survival on lettuce growing in the greenhouse was lower than on lettuce growing in a controlled growth chamber. Daily irrigation of the plant leaves had no influence on the epiphytic pathogen populations if the relative humidity was held at 80 %, but in greenhouse conditions it significantly prolonged their survival. Highest pathogen levels were observed on the inner, folded leaves of nearly mature plants, these are the leaves that are preferable consumed.

Also the effect of prior residence of the pathogens in irrigation water (and thus the exposure to nutrient stress) on the capacity for *E. coli* O157:H7 to attach onto lettuce leaves was investigated. The attachment of these stressed pathogens was compared with freshly cultured pathogens. We could show that for *E. coli* O157:H7 the attachment ratio for the stressed, but still alive, cells was about the same as for the unstressed, freshly grown cells. The results also indicated that standard laboratory solutions may not be the best to simulate stressed cells in irrigation water, in which the bacteria may behave significantly differently.

Furthermore, to improve the understanding about the underlying genetic mechanisms that the pathogens use during their survival on fresh produce, gene expression experiments of *E. coli* O157:H7 Sakai were performed when attached to growing butterhead lettuce plants. Our findings revealed that the pathogen actively interacts with the plant environment by adapting its metabolism and responding to oxidative stress. Consequently, the pathogen may have acquired enhanced resistance against oxidative stress and cross-protection against various other stresses. On the other hand, a decrease in the expression of the Shiga toxin and several other virulence genes was observed. Further research, is needed to investigate how these adaptations may affect the pathogen's subsequent survival and virulence during processing and consumption.

Samenvatting

Voor een gezonde en evenwichtige voeding wordt de consumptie van groenten en fruit aangemoedigd. De laatste jaren wordt evenwel ook een toename vastgesteld in het aantal uitbraken geassocieerd met groenten en fruit. De meeste uitbraken werden veroorzaakt door *Salmonella* en *Escherichia coli* O157:H7 (*E. coli* O157:H7), twee ziekteverwekkende bacteriën die voornamelijk voedselinfecties veroorzaken na de consumptie van besmet voedsel van dierlijke oorsprong zoals vlees, eieren en zuivel.

Hoewel het probleem voornamelijk in de Verenigde Staten voorkomt, komen uitbraken in Europa ook steeds meer voor. Heel recent, tijdens het verloop van deze studie, brak één van de grootste uitbraken uit in Duitsland en Frankrijk. De uitbraak, die door de media ook wel de 'EHEC crisis' werd genoemd, was hoogstwaarschijnlijk te linken aan de consumptie van fenegriek scheuten die besmet waren met *E. coli* O104:H4, een minder gekende ziekteverwekkende *E. coli* serovar. De uitbraak had zeer zware gevolgen, zowel voor de volksgezondheid als op economisch vlak en onderlijnde het belang om de risicofactoren voor de introductie en overleving van dergelijke ziekteverwekkers in de primaire productie van groenten en fruit te onderzoeken.

Voor deze studie werd kropsla, ook botersla genoemd, gekozen als model plant. Enerzijds omdat het gewas economisch belangrijk is voor België en anderzijds omdat er voor deze typische Noord-Europese slavariëteit nog niet veel kennis is over mogelijke besmetting met deze ziekteverwekkers. De focus van het werk lag op het onderzoeken van factoren die besmetting kunnen veroorzaken tijdens de teelt van het gewas door gebruik te maken van artificieel besmetten van zaden, irrigatiewater of groeiende slaplanten. Anderzijds werd er ook aandacht besteed om een natuurlijk besmetting zo goed mogelijk na te bootsten en te begrijpen. Daarom werd ook het gedrag van gestresseerde bacteriën onderzocht.

Een eerste doel bestond erin om na te gaan in hoeverre slazaden en irrigatiewater mogelijke insleeproutes vormen voor *Salmonella* en *E. coli* O157:H7 gedurende de productie van botersla in de serre. Daarom werd de overleving van vier verschillende bacteriële stammen onderzocht i) op slazaden gedurende een periode van twee jaar en ii) in vijf verschillende irrigatiewater stalen gedurende een periode van twee weken. Onze resultaten bevestigden dat zowel zaden als irrigatiewater een mogelijke bron van besmetting kunnen vormen. Zowel *E. coli* O157:H7 als *Salmonella* waren in staat om gedurende twee jaar te overleven op de slazaden. Wanneer deze zaden, na twee jaar, werden gekiemd, waren beide ziekteverwekkers in staat om terug in aantal toe te nemen op de kiemplantjes. *Salmonella* overleefde beter dan *E. coli* O157:H7 op de zaden. De bacteriën waren ook in staat om in de verschillende irrigatiewaterstalen te overleven, hun overleving varieerde afhankelijk van de stam en was beter bij lagere temperatuur (4°C) dan bij hogere (20°C).

Maar het belangrijkste resultaat was dat de overleving van de pathogeen zeer sterk verschilde tussen de verschillende stalen van Belgische slaproducenten. De waterstalen vertoonden een hoge variabiliteit in chemische en bacteriologische samenstelling en verschilden sterk in capaciteit om de ziekteverwerker over te brengen op de slaplanten omdat het overlevingsprofiel van de pathogeen verschillend was in elk waterstaal.

Een tweede doel van de studie bestond eruit om meer inzicht en kennis te verwerven over het gedrag van de twee ziekteverwekkende bacteriën gedurende de groeicyclus van de sla. De bacteriën werden op de bladeren van slaplanten aangebracht en vervolgens werden drie verschillende aspecten onderzocht: overleving, aanhechting en genexpressie. De overleving van de bacteriën op de slaplanten werd zowel in een groeikamer als in een serre onderzocht. De invloed van het kropstadium, de ouderdom van de slabladeren (of de positie van de bladeren in de slakrop) en het al dan niet irrigeren (op de plant zelf) werd nagegaan op zowel jonge en bijna oogstklare slaplanten. Onze resultaten toonden aan dat wanneer de bacteriën op de planten werden geïntroduceerd, ze in staat waren om te overleven op de bladeren van de plant. De overleving van de bacteriën en de groei van de bacteriën op de slabladeren was zeer variabel en deze variatie hing af van het groeistadium van de plant en bleek ook sterk af te hangen van de omgevingscondities, in het bijzonder de relatieve vochtigheid. Hierdoor was de overleving van de bacteriën op planten in de serre minder goed dan wanneer de planten in een groeikamer met gecontroleerde temperatuur en relatieve vochtigheid werden opgegroeid. Dagelijks irrigeren van de planten had geen invloed op de overleving van de pathogeen populaties op het blad wanneer de relatieve vochtigheid op 80% werd gehouden, maar in de serre kon het de overleving van de bacteriën sterk verlengen. De hoogste niveaus aan pathogenen werden bereikt op de binnenste gevouwen blaadjes in de slakrop, dit zijn het blaadjes die het liefst geconsumeerd worden.

Er werd ook nagegaan in hoeverre het verblijf van de pathogenen in irrigatie water (en dus ook het blootstellen van de pathogeen aan nutriënten tekort/stress) een invloed heeft op de aanhechtingscapaciteiten van *E. coli* O157:H7 aan slabladeren. We konden aantonen dan de aanhechting van levende, maar gestresseerde bacteriën niet substantieel verschilde van ongestresseerde bacteriën. De resultaten toonden ook aan dat het gedrag van deze bacteriën in standaard laboratorium oplossingen niet altijd het gedrag van de bacteriën in irrigatiewater uit de praktijk correct weerspiegelen.

Ten slotte werd er getracht om een beter inzicht te verwerven in de onderliggende genetische mechanismen die de bacteriën gebruiken om te overleven op groenten en fruit. De resultaten van deze genexpressie experimenten brachten aan het licht dat *E. coli* O157:H7 Sakai actief interageert

met de plant door zijn metabolisme aan te passen en door te reageren op aanwezige oxidatieve stress. Ten gevolge hiervan is het mogelijk dat de bacteriën een betere resistentie ontwikkelen tegen deze stress maar bovendien ook mogelijk kruisresistentie kunnen ontwikkelen ten opzichte van andere stressoren. Anderzijds werd er een afname in de expressie van verschillende virulentiegenen waargenomen waaronder de Shiga toxines. Verder onderzoek zal moeten uitwijzen in hoeverre deze aanpassingen de overleving en virulentie van de ziekteverwekkers gedurende de verwerking en consumptie van de sla kan beïnvloeden.

Curriculum Vitae

Personal information

Name	Inge Van der Linden
Date of birth	October 3, 1983
Place of birth	Sint-Niklaas
Nationality	Belgian
Contact home	Kleempendorp 11 9070 Heusden Belgium +32(0)486 656 855
Contact work	Brusselsesteenweg 370 9820 Melle Belgium +32(0)9 272 30 76

Education

2005 – 2008	Master after Master, Sustainable Development and Human Ecology, option 'Milieucöordinator A', Faculty of Medicins, Vrije Universiteit Brussel
2006 – 2007	Specific teachers training, Faculty of Psychology and Educational Sciences, Ghent University
2001 – 2005	MSc in Biology – Botany, Faculty of Sciences, Ghent University
1994 – 2000	Secondary school, Latin – Science, Instituut Heilige Familie, Sint-Niklaas

Professional experience:

2014 – present	Scientist at the Institute for Agricultural and Fisheries Research (ILVO), Food Science Unit, Food safety, Segeri project
2013 – 2014	Scientist at Ghent University, Department of Food Safety and Food Quality, Laboratory of Food Microbiology and Food Preservation, Veg-i-trade project
2009 – 2013	PhD Student at the Institute for Agricultural and Fisheries Research (ILVO), Plant Science Unit, Crop protection & Food Science Unit, Food safety
2008 – 2009	Project attaché, Federale Overheidsdienst veiligheid van de voedselketen en leefmilieu, Brussel, Belgium
2006 – 2008	Scientific responsible, Deroose Plants NV, Evergem, Belgium

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Van der Linden I., Cottyn B., Vlaemynck G., Baert L., Uyttendaele M., Heyndrickx M. & Maes M. (2011) The survival of *E. coli* O157 and *Salmonella* in irrigation water and the influence on their subsequent survival on butterhead lettuce leaves. Sixteenth Conference on Food Microbiology, Brussels (BE), Poster, 22-23/09/2011 - Biomérieux Poster Award (shared first place)

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Van der Linden I., Cottyn B., Vlaemynck G., Uyttendaele M., Maes M & Heyndrickx M. (2012) The long-term survival of *Escherichia coli* O157:H7 and *Salmonella* on lettuce seeds, and their subsequent survival and growth on germinating sprouts. IAFP Annual Meeting 2012, Providence (USA), Technical Presentation, 22-25/07/2012

Van der Linden I., Cottyn B., Vlaemynck G., Uyttendaele M., Maes M. & Heyndrickx M. (2012) Groei en overleving van *E. coli* O157 en *Salmonella* in het teeltproces van botersla. Seventeenth Conference on Food Microbiology, Brussels (BE), Invited Talk, 20-21/09/2012

Van der Linden I., Cottyn B., Uyttendaele M.,Vlaemynck G., Heyndrickx M., Maes M. & Holden N. (2013) Gene expression in *Escherichia coli* O157:H7 present as epiphyte during cultivation of butterhead lettuce. Eighteenth Conference on Food Microbiology, Brussels (BE), Poster, 12-13/09/2013, Biomérieux Poster Award

Van der Linden I., Cottyn B., Uyttendaele M.,Vlaemynck G., Heyndrickx M. & Maes M. (2014) The survival and persistence of *E. coli* O157 and *Salmonella* during butterhead lettuce production. KNPV Werkgroep Fytobacteriologie, Technical Presentation, 06-03-2014

Conference attendance without contribution

61th International Symposium on Crop Protection. Gent, May 19, 2009.

Courses and workshops

Advanced Academic English, writing, Ghent University

ANOVA, Prof. M. Ysebaert, Ghent University

Supervision of undergraduate students

Ewout Ruysbergh & Lien Van De Wielle. Internship (1 month). Bachelor students Bio-engineering, Faculty of Bio-engineering, UGent, Gent

Olivier Andries . De overleving van de zoönotische pathogenen *Salmonella* en *Escherichia coli* O157:H7 in water en op botersla (*Lactuca sativa*). Bachelor in Chemistry option Biochemistry. Academic Year 2010-2011. KaHo Sint-Lieven Gent.

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