Prevalence, behavior and risk assessment of *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* on basil leaves and strawberries

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List of abbreviations

A/E	attaching and effacing
aggR	activator aggregative adherence regulator gene
approx.	approximately
BCIG	5-bromo-4-chloro-3-indolyl-b-D-glucuronide
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
CBI	Center for the Promotion of Imports from developing countries
CECT	Spanish Type Culture Collection
cfu	colony forming units
C.I.	confidence interval
CSPI	Center for Science in the Public Interest
CT	threshold cycle
CT-SMAC	Cefixime Tellurite Sorbitol MacConkey
d	day(s)
DAEC	Diffusely Adherent *Escherichia coli*
DNA	deoxyribonucleic acid
eae	intimin-coding gene
EAEC	Enteroaggregative *Escherichia coli*
EAHEC	Enteroaggregative Hemorrhagic *Escherichia coli*
EEA	European Economic Area
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxinogenic *Escherichia coli*
EU	European Union
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
FoAO	food of animal origin
FoNAO	food of non-animal origin
FSMA	FDA Food Safety Modernization Act
GAP	good agricultural practices

GHP	good hygiene practices
GUD	β-glucuronidase
H	flagellar (H) antigen
HC	haemorrhagic colitis
PHE	Public Health England
HUS	haemolytic-uraemic syndrome
IMS	immunomagnetic separation
iroB	fur-regulated gene of *Salmonella*
IPTG	isopropyl-β-D-thiogalactopyranoside
ICMSF	International Commision on Microbiological Specifications for Foods
ISO	International Organization for Standardization
LEE	locus of enterocyte effacement
LFMFP	Laboratory of Food Microbiology and Food Preservation
MAC	MacConkey agar
MALDI-TOF	matrix assisted laser desorption ionization - time of flight
MKTTn	Muller-Kauffmann Tetrathionate-Novobiocin
MSRV	modified semi-solid Rappaport-Vassiliadis agar
mTSB+N	modified Tryptone Soya Broth with novobiocin
NaCl	sodium chloride
NSCFS	Norwegian Scientific Committee for Food Safety
O	somatic polysaccharide, O-antigen
PCR	polymerase chain reaction
PPS	Physiological Peptone Salt solution
QMRA	quantitative microbiological risk assessment
RASFF	Rapid Alert System for Food and Feed
REC2	Rapid'*E. coli*2
rpm	rounds per minute
RTE	ready-to-eat
RVS	Rappaport-Vassiliadis with Soya
SB	*Salmonella Brilliance*
SMAC	Sorbitol MacConkey
sQMRA	swift quantitative microbiological risk assessment
STEC	Shiga toxin-producing *Escherichia coli*
stx	Shiga toxin-coding gene
TBX	Tryptone Bile X-glucuronide

TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
UK	United Kingdom
US	United States
US FDA	United States Food and Drug Administration
USDA	United States Department of Agriculture
UV	ultra violet
VRBG	Violet Red Bile Glucose
VRBL	Violet Red Bile Lactose
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XLD	Xylose-Lysine-Desoxycholate

SUMMARY

Several factors potentially playing a role in foodborne outbreaks related to enteric bacteria in fresh produce are investigated in this PhD thesis. Prevalence and behavior of enteric pathogens *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC) on basil and strawberries were experimentally researched and these insights were incorporated in an exposure assessment to gain additional information by calculations. This PhD starts with a literature review of the research area (CHAPTER 1) and ends with a discussion, synthesis of the obtained results and insights and finishes with a conclusion (CHAPTER 7). The performed experimental work is divided in three parts.

1. **Evaluation of a high-throughput multi-screening PCR method and conventional culture methods for detection of *Salmonella* and STEC on basil, strawberries and a lettuce mix.**

The first objective was to determine the suitability of a real-time PCR screening assay, namely GeneDisc multiplex PCR, and culture methods described in ISO 6579:2002 and ISO 16654:2001 for detection of *Salmonella* and STEC on basil, strawberries and a lettuce mix (CHAPTER 2). Therefore, sample replicates were artificially inoculated with *Salmonella* Thompson and STEC O157 or O26 and analyzed after one and five days of storage (strawberries and lettuce at 7°C and basil at 10°C). Overall, this study indicates the ability of PCR based screening methods for reproducible multi-detection of low numbers (10-70 cfu/25g) of STEC (*stx1*, *stx2*, *eae*) and *Salmonella* (*iroB* gene) by PCR in this type of foods. However, for the basil samples, PCR needed twofold dilution of the DNA extract to overcome inhibition. In case of strawberries, reduced detection rates of *S.* Thompson and STEC O157 were observed after five days of storage, in particular for the lowest inoculum level. It was noted that on several occasions presumptive colonies on the selective agar media were difficult to find due to the growth of competitive microbiota. The use of an additional agar medium such as CHROMagar STEC (without IMS) improved the recovery rate of STEC.

2. Assessment of the prevalence and survival behavior of *Salmonella* and STEC on fresh herbs (exemplified by basil and coriander) and soft fruit (exemplified by strawberries)

In CHAPTER 3, the behavior of *Salmonella* and STEC was assessed on basil stored at 7°C, 15°C and 22°C and strawberries stored at 4°C, 10°C, 15°C and 22°C. Both *Salmonella* and *E. coli* O157:H7 showed a gradual decrease in numbers if inoculated on strawberries, with a similar reduction observed at 4°C, 10°C and 15°C (2 to 3 log units after 5 days). At 22°C strawberries were moldy after 2 or 4 days and a 1 to 2 log reduction of both pathogens had occurred. A restricted die-off (on average 1.0 log unit) and increase (on average < 0.5 log unit) of both pathogens occurred on basil leaves after 7 days storage at 7°C and 22°C respectively. On leafy greens, a comparable decrease as on basil was observed after 3 days at 7°C. At 22°C both pathogens increased to higher numbers on fresh-cut iceberg and butterhead lettuce (on average 1.0 log unit), probably due to the presence of exudates. But, by using spot inoculation, pathogen growth was rather limited probably due to minimized contact between the inoculated cells and cell exudates. Overall, it could be concluded that both pathogens survive on basil, strawberries and (fresh-cut) leafy greens during storage.

In CHAPTER 4, a microbiological sampling was conducted of three soil and three soilless strawberry production sites in Belgium in 2012. Strawberries and environmental samples (irrigation water and substrate/plastic cover of soil) were analyzed for the presence of *Salmonella,* STEC and generic *E. coli*. Hands of the pickers were analyzed for Enterobacteriaceae and *E. coli*. The sanitary quality of Belgian strawberries was evaluated as good: very few samples contained *E. coli* (2/72) and none were contaminated with *Salmonella* or STEC. However, environmental pressure was noticed for STEC because STEC (*stx* and *eae* genes) was detected by PCR in 11 out of 78 irrigation water samples and 2 out of 24 substrate samples. Subsequently, STEC O26 was isolated in 2 out of 11 PCR positive irrigation water samples and both PCR positive substrate samples. A follow-up study in one farm in 2013 indicated cattle as the most likely source of STEC contamination of irrigation water.

In CHAPTER 5, fresh pre-packed basil and coriander leaves present on the Belgian market were investigated for the presence of *Salmonella,* STEC, generic *E. coli* and coliforms. In total 592 samples were collected originating from Belgium, Israel and Cyprus during the period 2013-2014. Results indicate that fresh leafy herbs like basil and coriander sourced from different cultivation regions, may contain enteric pathogens. *Salmonella* was detected in 10 out of 592 samples (5 basil and 5 coriander), of which two samples were sourced from Israel and eight from Cyprus. STEC (*stx* and *eae* genes) was detected in 11 out of 592 samples (3 basil and 8 coriander) of which one originated from Belgium, four from Israel and six from Cyprus. No STEC were isolated by culture techniques, but in three samples a serotype (O26, O103 or O111) with its associated *eae*-variant (β or θ) were detected by PCR.

3. Exposure assessment and risk ranking of *Salmonella* and STEC on basil and strawberries compared to lettuce

In CHAPTER 6, an exposure assessment model was built in MS Excel with @Risk for a quantitative risk ranking for basil, strawberries and lettuce. Input information was collected based on research information of the previous chapters, additional data collection from other research groups of the Veg-i-Trade project, literature information and expert discussion. Lettuce was considered as a reference commodity to assess how niche products such as basil and strawberries, which are increasingly popular in culinary preparations in the West-European diet, rank compared to lettuce. Two European countries (Belgium and Spain) were included in these calculations to evaluate consumer behavior practices and consumption behavior. The probabilistic output of the model resulted in a distribution of the number of contaminated portions basil, strawberries or lettuce to which consumers are exposed per year. The highest number of contaminated portions per year was found for basil (five to nine portions for a daily consumption), followed by lettuce and strawberries. This is in particular due to the higher prevalence of enteric pathogens found on basil leaves (CHAPTER 5), their better survival during storage (CHAPTER 3) and less occurrence of consumers washing the basil leaves before consumption. The lowest exposure was observed for strawberries in the Spanish

situation mainly due to the lower consumption frequency of strawberries but in combination also with higher die-off of the enteric pathogens on strawberries versus basil leaves or lettuce. From the scenario analysis it was noted that the main driving force in exposure to contaminated portions is the prevalence of pathogens in these fresh produce items, a moderate effect of consumption frequency and to a minor extent the consumer behavior (such as time/temperature of storage or washing practices). This quantitative risk ranking calculation demonstrates that also niche products such as basil leaves can have an impact on public health equal or higher than more convenient consumed leafy greens and also need priority in food safety protection, monitoring and surveillance whereas strawberries indeed are confirmed to be quite safe fruit products.

SAMENVATTING

Verschillende factoren die mogelijks een rol spelen in voedselgebonden uitbraken, veroorzaakt door intestinale bacteriën in verse groenten en fruit, werden onderzocht in dit doctoraatswerk. De prevalentie en het overlevingsgedrag van de pathogenen *Salmonella* en Shiga toxine producerende *Escherichia coli* (STEC) op basilicum en aardbeien werden experimenteel onderzocht en vervolgens geïncorporeerd in een blootstellingsbeoordeling om extra (mathematisch georiënteerde) informatie te verwerven. Het doctoraat begint met een overzicht van de literatuur m.b.t. het onderzoeksonderwerp (HOOFDSTUK 1) en eindigt met een discussie, synthese en inzicht van de verkregen resultaten en een conclusie (HOOFDSTUK 7). Het experimentele werk kan opgedeeld worden in 3 stukken.

1. **Evaluatie van een 'high throughput' multi-screening PCR methode en klassieke cultuurmethoden voor de detectie van *Salmonella* en STEC op basilicum, aardbeien en een slamix.**

Een eerste doelstelling van dit werk was om de geschiktheid van een real-time PCR screening, namelijk GeneDisc multiplex PCR, en cultuurmethoden, beschreven in ISO 6579:2002 en ISO 16654:2001, voor de detectie van *Salmonella* en STEC op basilicum, aardbeien en een slamix te bepalen (HOOFDSTUK 2). Stalen werden artificieel geïnoculeerd met *Salmonella* Thompson en STEC O157 of O26 en geanalyseerd na één of vijf dagen bewaren (aardbeien en sla bij 7°C en basilicum bij 10°C). Het onderzoek toont aan dat de multiplex PCR een reproduceerbare multi-detectiemethode is voor lage aantallen (10-70 cfu/25 g) van *Salmonella* (*iroB* gene) en STEC (*stx1*, *stx2*, *eae*) op deze levensmiddelen. Echter, een tweevoudige verdunning van het DNA-extract was nodig om inhibitie van de PCR reactie bij basilicumstalen te voorkomen. In het geval van aardbeien werd een verlaagde detectiegraad van *S.* Thompson en STEC O157 waargenomen na 5 dagen bewaren, in het bijzonder voor de laagste inoculum niveaus. In verschillende gevallen werd de groei van competitieve microbiota opgemerkt die het vinden van presumptieve kolonies op selectieve agar media belemmerde. De recovery van STEC verbeterde bij het gebruik van een extra agar medium, zoals CHROMagar STEC (zonder IMS).

2. Beoordeling van de prevalentie en het overlevingsgedrag van *Salmonella* en STEC op verse kruiden (geïllustreerd door basilicum en koriander) en zacht fruit (geïllustreerd door aardbeien)

In HOOFDSTUK 3 werd het gedrag bepaald van *Salmonella* en STEC op basilicum bewaard bij 7°C, 15°C en 22°C en aardbeien bewaard bij 4°C, 10°C, 15°C en 22°C. Op aardbeien werd, zowel voor *Salmonella* als voor *E. coli* O157:H7, een graduele daling in aantallen waargenomen. Een gelijkaardige daling werd vastgesteld bij 4°C, 10°C en 15°C (2 tot 3 log eenheden na 5 dagen bewaren). Bij 22°C waren aardbeien na 2 of 4 dagen beschimmeld en een 1 tot 2 log daling van beide pathogenen werd waargenomen. Na 7 dagen bewaren bij 7°C en 22°C werd een beperkte afdoding (gemiddeld 1 log eenheid) en groei (gemiddeld < 0.5 log eenheid), respectievelijk, van beide pathogenen op basilicum blaadjes vastgesteld. Op verschillende bladgroenten werd een vergelijkbare daling als op basilicum waargenomen na 3 dagen bewaren bij 7°C. Op vers gesneden ijsbergsla en botersla groeiden beide pathogenen tot hogere aantallen (gemiddeld 1 log eenheid) na bewaren bij 22°C, waarschijnlijk door de aanwezigheid van celexudaten. Maar door het gebruik van spot inoculatie was de groei van pathogenen eerder beperkt, mogelijks omdat het contact tussen het inoculeerde cellen en de celexudaten geminimaliseerd werd. Er kan besloten worden dat beide pathogenen kunnen overleven op basilicum, aardbeien en andere bladgroenten tijdens het bewaren.

In HOOFDSTUK 4 werd een microbiologische staalname uitgevoerd in de aardbeienteelt (drie grondteelten en drie substraatteelten) in België (2012), waarbij zowel aardbeien als omgevingsstalen (irrigatiewater en substraat of plastiek cover van de grond) geanalyseerd werden voor de aanwezigheid van *Salmonella*, STEC en generieke *E. coli*. Handen van plukkers werden geanalyseerd voor Enterobacteriaceae en *E. coli*. De microbiologische kwaliteit van Belgische aardbeien werd als goed geëvalueerd: weinig stalen bevatten *E. coli* (2/72) en geen enkel was besmet met *Salmonella* of STEC. Er werd echter omgevingsdruk voor STEC vastgesteld omdat STEC (*stx* en *eae* genen) gedetecteerd werd door middel van PCR in 11 van de 78 irrigatiewater stalen en 2 van de 24 substraatstalen. Vervolgens werd STEC O26 geïsoleerd uit 2 van de 11 PCR positieve irrigatiewater stalen en in beide PCR positieve substraatstalen. Gedurende een follow-up studie van één bedrijf in 2013 werd vastgesteld dat het (vlees)vee, aanwezig

op het aardbeienbedrijf, hoogstwaarschijnlijk de bron van contaminatie van STEC was in het irrigatiewater.

In HOOFDSTUK 5 werden vers, verpakte basilicum en koriander blaadjes van de Belgische markt onderzocht voor de aanwezigheid van *Salmonella*, STEC, generieke *E. coli* en coliformen. In het totaal werden 592 stalen, afkomstig uit België, Israël en Cyprus, verzameld gedurende 2013-2014. Resultaten toonden aan dat verse bladkruiden, zoals basilicum en koriander afkomstig uit verschillende teeltgebieden, intestinale pathogenen kunnen bevatten. *Salmonella* werd gedetecteerd in 10 van de 592 stalen (5 basilicum, 5 koriander), waarbij 2 stalen afkomstig waren van Israël en acht stalen van Cyprus. STEC (*stx* en *eae* genen) werd gedetecteerd in 11 van de 592 stalen (3 basilicum, 8 koriander), waarbij één staal afkomstig was van België, 4 stalen van Israël en zes stalen van Cyprus. STEC werd in geen enkel staal geïsoleerd m.b.v. cultuurmethoden, maar in drie stalen werd een serotype (O26, O103 en O111) met de geassocieerde *eae*-variant (β or θ) gedetecteerd m.b.v. PCR.

3. Blootstellingsbeoordeling en risicorangschikking van *Salmonella* en STEC op basilicum en aardbeien in vergelijking met sla

In HOOFDSTUK 6 werd een kwantitatief risicomodel opgebouwd in MS Excel met @Risk om de blootstelling te beoordelen en om de kwantitatieve risicorangschikking van basilicum, aardbeien en sla te maken. Input data werd verzameld uit voorgaande hoofdstukken, andere onderzoeksgroepen van het Veg-i-Trade project, wetenschappelijke literatuur en expert discussies. Sla werd beschouwd als een referentie om na te gaan hoe niche producten, zoals basilicum en aardbeien, die steeds populairder worden in West-Europese maaltijden, zich rangschikken t.o.v. de referentie sla. Twee Europese landen, België en Spanje, werden opgenomen in de berekeningen om de invloed van consumentengedrag en consumptie te evalueren. De probabilistische output van het model was een distributie van het aantal gecontamineerde porties basilicum, aardbeien en sla per jaar waar consumenten aan blootgesteld worden. Het grootste aantal gecontamineerde porties per jaar werd gevonden voor basilicum (vijf tot negen porties bij een dagelijkse consumptie), gevolgd door sla en aardbeien,

voornamelijk door de hogere prevalentie van intestinale pathogenen, in the bijzonder *Salmonella*, op basilicum (HOOFDSTUK 5) en hun betere overleving op basilicum gedurende bewaren (HOOFDSTUK 3) en doordat basilicum minder gewassen wordt voor consumptie door de consumenten. De laagste blootstelling werd vastgesteld voor aardbeien in de Spaanse situatie, voornamelijk door een lager consumptiefrequentie van aardbeien in combinatie met een grotere afdoding van intestinale pathogenen op aardbeien in vergelijking met basilicum en sla. Uit de scenarioanalyse werd vastgesteld dat de prevalentie van pathogenen op verse groenten en fruit de belangrijkste factor is voor het berekenen van de blootstelling, d.w.z. het aantal gecontamineerde porties. Een middelmatig effect werd waargenomen voor de consumptiefrequentie op de berekeningen, terwijl het consumentengedrag (bv. tijd/temperatuur tijdens bewaren en wassen) slechts een beperkte invloed had. De kwantitatieve risicorangschikking demonstreert dat ook niche producten, zoals basilicum, een impact kunnen hebben op de volksgezondheid die gelijk of hoger is dan bladgroenten. Bovendien zouden niche producten, zoals basilicum, een prioriteit moeten zijn op vlak van voedselveiligheid, monitoring en surveillance, terwijl de status van aardbeien als een veilig product werd bevestigd.

OBJECTIVES AND OUTLINE

The overall objective of the present PhD study is to gain insight in the prevalence and behavior of enteric pathogens *Salmonella* and Shiga toxin-producing *Escherichia coli* on basil and strawberries and to elaborate an exposure assessment.

The growing demand for convenient, healthy and tasty food and dietary guidelines on the consumption of at least five portions of fruit and vegetables per day resulted in increased production and trade of fresh fruit and vegetables. However, international, European and national concerns have emerged with regard to the safety of fresh produce in response to recent outbreaks and reported hazards, such as norovirus and pathogenic *E. coli*, being linked to fresh produce and derived food products. Within this context of concerns of fresh produce and its associated hazards, the EU FP7 Veg-i-Trade project has been developed, to assess the impact of climate change and globalization on food safety of fresh produce including both microbiological and chemical hazards. The Veg-i-Trade project was a collaboration between twenty three international partners from universities, research institutes, small and medium enterprises and large industrial partners. In the project climate change was exemplified by different European regions, whereby Norway represented Northern Europe, Belgium middle Europe and Spain Southern Europe. The present study was situated within work package 6 of the Veg-i-Trade project: the risk assessment of microbiological food safety of fresh produce and derived products, more in particular focusing on the case studies of basil and strawberries and enteric pathogens *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC). These fresh produce commodities were, next to lettuce and baby leaves, selected as fresh produce items under study due to a combination of i) representing economically important products, both in production volumes and trade (intra-EU and import into Europe from third countries); ii) being (increasingly) part of the European diet and culinary traditions and iii) their association with bacterial foodborne outbreaks either in Europe or in other regions of the world. Therefore, information related to the prevalence and behavior during storage at different temperatures of both pathogens *Salmonella* and STEC on basil and strawberries was collected. To do so, first detection methods needed some evaluation for their appropriateness in use for these specific food products. Subsequently, the obtained data on prevalence and behavior were used as input in the comparative exposure assessment model.

Overall, the present research is divided into three objectives and seven chapters as shown in the overview below (Figure 1). The first CHAPTER gives a general introduction to the work, whereas in the last CHAPTER a general discussion of the research is presented.

The **first objective** (CHAPTER 2) of the research was the evaluation of a rapid molecular method, i.e. multiplex real-time polymerase chain reaction (PCR), and culture methods for their performance in the detection of *Salmonella* (exemplified by *S.* Thompson) and STEC (exemplified by *E. coli* O157 and O26) on basil and strawberries. Also lettuce was included in this chapter to check the performance of the methods, because (i) also lettuce is a commodity which was investigated in the Veg-i-Trade project, (ii) inhibition of the PCR-reaction, due to the presence of chlorophyll, is known and (iii) to allow comparison of the performance of the detection methods in case of basil and strawberries. The GeneDisc detection kit of Pall Technologies, was applied for the molecular detection. In addition, the suitability of the detection by classical plating of *Salmonella* and STEC was determined.

The **second objective** was the determination of the behavior of *Salmonella* and STEC on basil and strawberries during storage at different temperatures and the collection of information on the prevalence of these pathogens. Thus, in CHAPTER 3 the behavior of the pathogens on basil and strawberries during simulated home storage at various reasonably foreseen temperatures was monitored by plating on suitable agar media. Moreover, the behavior of these enteric pathogens on basil was compared to the behavior on lettuce and other leafy greens. The presence of *Salmonella*, STEC and generic *E. coli* on basil leaves is demonstrated in CHAPTER 5 whereas the focus in CHAPTER 4 was on sampling of strawberries and the strawberries' production environment. In the latter chapter, it was also the intention to look into the risk factors at primary production affecting the introduction of enteric bacterial pathogens on fresh produce such as strawberries. For the detection of *Salmonella* and STEC on basil leaves and strawberries (and environmental samples), the GeneDisc kit, evaluated in CHAPTER 2, was used to analyze samples for the presence of the *iroB* gene and *stx1*, *stx2*, *eae* and *aggR* genes, respectively.

The **last objective** was to build a comparative retail to fork exposure assessment model (CHAPTER 6). Data collected in CHAPTER 3, 4 and 5 were used as input for the exposure assessment model. The model aimed at showing the effect of storage time and temperature and washing practices by consumers, incorporating specific consumer handling practices and consumption behavior, on the occurrence of *Salmonella* and *E. coli* O157 on basil and strawberries. Taking the measured prevalence of the pathogens into account, a risk ranking is made aimed at comparing pathogens, commodities or regional consumption patterns in their potential vulnerability towards these microbiological hazards related to the consumption of fresh produce. In addition, butterhead lettuce was included in the model and considered as a reference item to assess how these other two niche products rank in reference to lettuce.

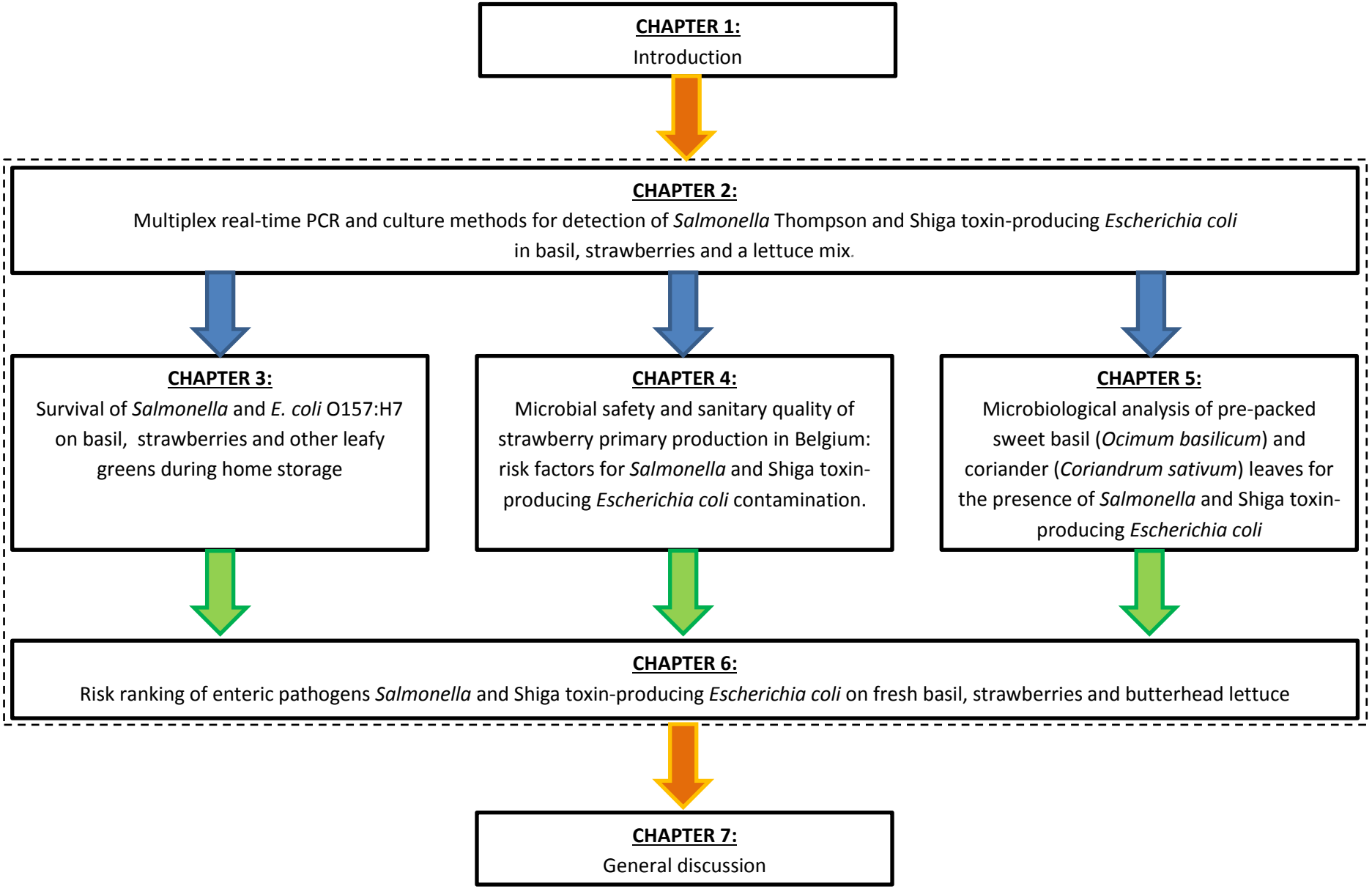

Figure 1: Overview of the different chapters of the present research

CHAPTER 1: Introduction

1.1 Fresh fruit and vegetables

Consumption of fresh fruit and vegetables continues to increase with the encouragement of (governmental) health agencies in many countries. They are important components of a healthy and balanced diet due to the presence of vitamins (e.g. C and E), minerals (e.g. folic acid, potassium and selenium), fibers and other dietary components (e.g. terpenes, phenols and flavonoids). A strong link was found between increased fruit and vegetable consumption and decreased risk of chronic diseases such as cancer, heart disease and stroke. Moreover, evidence for reducing the risk of cataracts, diverticulosis, chronic obstructive pulmonary disease and hypertension is emerging due to consumption of fruit and vegetables (Van Duyn & Pivonka, 2000). However, fruit and vegetables, in particular leafy green vegetables, that are consumed raw, have been associated with some notable outbreaks of microbial foodborne diseases (Berger et al., 2010).

1.1.1 Outbreaks related to (fresh) fruit and vegetables

Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* were observed as the most important cause of illness related to fresh produce. STEC has been linked to meat more often than to any other food product (Doyle et al., 2006; EFSA, 2013a). Severe STEC outbreaks have been traced to the consumption of contaminated fruit and vegetables including radish sprouts (Michino et al., 1999), fenugreek sprouts (Soon et al., 2013), pre-packaged spinach (Grant et al., 2008), lettuce (Ackers et al., 1998; Friesema et al., 2007; Soderstrom et al., 2008) and strawberries (Laidler et al., 2013). Similarly, while infection with *Salmonella* has mainly been linked to consumption of foods of animal origin, outbreaks have also been traced to tomatoes (Cummings et al., 2001; Greene et al., 2008), melons (Bowen et al., 2006; Munnoch et al., 2009), basil (Pakalniskiene et al., 2009; Pezzoli et al., 2008), alfalfa sprouts (Mahon et al., 1997; Werner et al., 2007), fresh cilantro (Campbell et al., 2001) and rucola lettuce (Nygard et al., 2008). Besides, the enteric pathogens STEC and *Salmonella*, fruit and vegetables have been linked to outbreaks associated with *Cyclospora* (e.g. basil, raspberries) (Ho et al., 2002; Lopez et al., 2001), *Shigella sonnei* (e.g. basil, parsley, lettuce) (Davis et al., 1988; Guzman-Herrador et al., 2011; Naimi et al., 2003), norovirus (e.g. frozen strawberries and raspberries) (Bernard et al., 2014; Cotterelle et al., 2005; Hjertqvist

et al., 2006) and hepatitis A virus (e.g. lettuce and frozen strawberries) (Hutin et al., 1999; Rosenblum et al., 1990).

In the US, leafy greens were the first one of the top 10 of riskiest foods regulated by the Food and Drug Administration (FDA), which is responsible for all produce, seafood, shell eggs and dairy products (meat, poultry and egg products excluded as they are regulated by United States Department of Agriculture (USDA)) (Figure 2). These ten products account for nearly 40% of all foodborne outbreaks linked to FDA-regulated foods (CSPI, 2014).

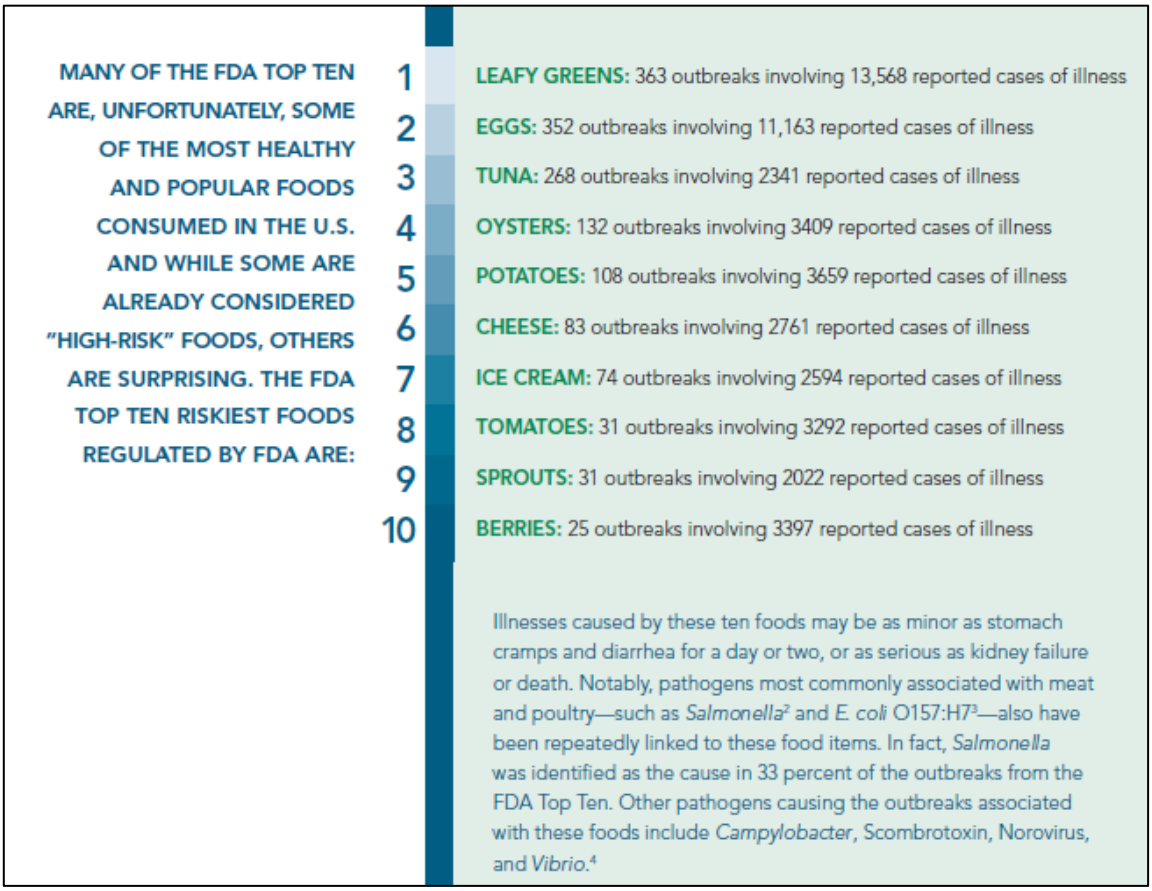

Figure 2: The riskiest foods regulated by the U.S. Food and Drug Administration since 1990 – Findings (CSPI, 2014)

Also in the EU, there was an increase in the number of reported outbreaks, cases, deaths and hospitalizations associated with food of non-animal origin (FoNAO) from 2008 to 2011. Amongst all foodborne outbreaks reported, where food of either non-animal or animal origin were implicated (between 2007 and 2011), FoNAO were associated with 10% of the outbreaks, 26% of the cases, 35% of the hospitalizations and 46% of the deaths (EFSA, 2013b). There is a general tendency for the outbreaks associated with FoNAO to involve

more cases than those associated with food of animal origin (FoAO), but to be less severe in that there is a lower proportion of hospitalisations and deaths. In order to provide a general overview of the most frequently reported combinations of foodborne pathogen and FoNAO, Table 1 provides a summary of the food type and pathogen group combinations for which more than one outbreak was reported between 2007-2011 with the aggregated numbers of human cases, hospitalisations and deaths. As in the US, leafy greens are among the most frequently reported implicated foodstuff (EFSA, 2013b).

Table 1: Number of outbreaks reported for most frequent[a] combinations of foodborne pathogen and FoNAO[b] (2007-2011) (EFSA, 2013b)

Foodstuff implicated[c]	Causative agent	Number of outbreaks	Human cases	Number of cases hospitalized	Deaths
Raspberries	Norovirus	27	913	3	0
Leafy greens eaten raw as salads	Norovirus	24	657	1	0
Sprouted seeds	*Salmonella*	11	521	76	1
Leafy greens eaten raw as salads	*Salmonella*	7	438	29	0
Spices and dry herbs	*Bacillus*	7	343	0	0
Fresh pods, legumes and grain	*Shigella*	4	268	3	0
Sprouted seeds	STEC	3	3830	2381	53
Bulb and stem vegetables	Norovirus	2	18	0	0

(a) 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.

(b) 136 foodborne outbreaks have been excluded due to the fact that the implicated foodstuffs were composite products. In total 219 foodborne outbreaks associated to FoNAO were reported from 2007-2011. Data for the year 2011, extracted by EFSA's Unit on Biological Monitoring on 24/09/2012, is preliminary, until the publication of "The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Foodborne Outbreaks in 2011".

(c) Outbreaks implicating: (i) 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.

1.1.2 Transmission of enteric pathogens to fresh produce

Fruit and vegetables can become contaminated with foodborne pathogens, such as *Salmonella* or STEC, during cultivation, during and after harvest, at processing, during distribution and at the consumer's home, through (raw manure fertilized) soil, irrigation and processing water, insects, wild and domestic animals, equipment and human handling (Beuchat, 2002; Brackett, 1999; Brandl, 2006; Gil et al., 2015). Contamination of fresh produce frequently occurs through agricultural practices, such as application of polluted irrigation water or fertilization with manure and sewage sludge (Nguyenthe & Carlin, 1994) (Figure 3).

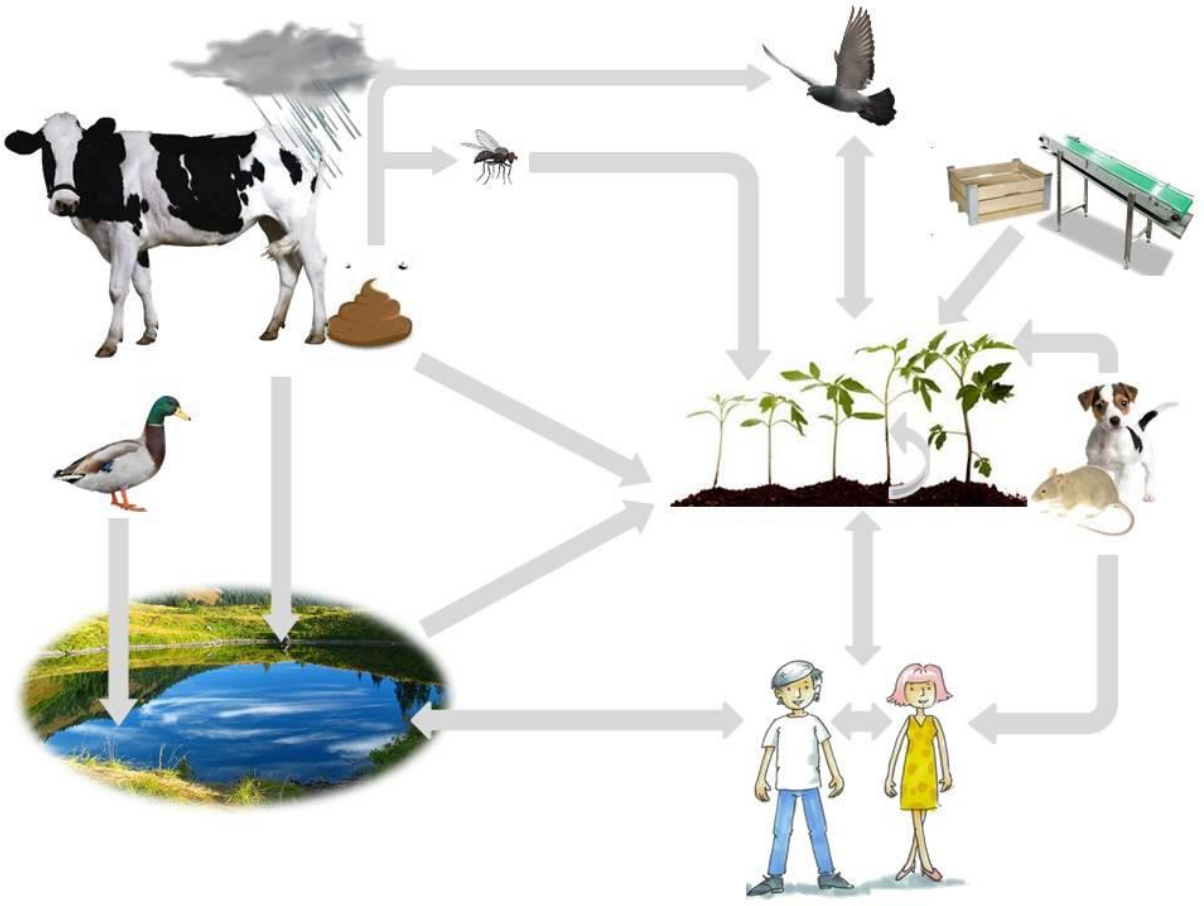

Figure 3: Overview of possible transmission routes of enteric pathogens

Water, used for irrigation or for pesticide application, is an important source of contamination in the field. For example, irrigation water was suggested as the contamination source of basil with *Salmonella* (Pakalniskiene et al., 2009). The risk of transmission of pathogens to the crops (and subsequently to the consumer) via the irrigation

water is influenced by the source of the irrigation water, the initial contamination level of the water, the method of irrigation and the persistence of pathogens in the water but also in the soil and on the crop. Borehole water is most of the time of good microbial quality, while surface water (e.g. ponds) is more susceptible to contamination by run-off water from animal pastures or by fecal droppings of wildlife and birds (De Roever, 1998; Steele & Odumeru, 2004). However, contamination of the fresh produce by irrigation depends also on the type of irrigation system used (drip versus overhead (sprinkler, spray) irrigation). Drip irrigation has a lower likelihood of transmitting pathogens from contaminated water to fresh produce as the water is not directly introduced on (the edible part of) the plant (Brackett, 1999). Mitra et al. (2009) reported that no internalization of *E. coli* O157:H7 was observed in spinach plants from contaminated soil, whereas uptake and internalization was found in spinach leaves after contaminated water was dropped on the spinach leaves. Nevertheless, contact of the edible part with contaminated soil can also lead to contamination (De Roever, 1998). In Kisluk et al. (2013), it was observed that, when applying drip irrigation, water harboring an extremely high concentration (i.e. 8.5 log cfu/ml) of *Salmonella* was needed to contaminate basil leaves. Furthermore, plants grown in hydroponic systems have been shown to have lower microbial contamination (e.g. lactic acid bacteria and total coliforms) than plants grown in soil (Selma et al., 2012). Lastly, the extent of contamination depends on the type of produce. Leafy green vegetables provide a large surface and have features to foster the attachment and entrapment of microorganisms (De Roever, 1998).

Manure or sewage sludge might be applied to fields in order to dispose animal waste and fertilize soils. Fertilized soil can contaminate produce if manure or sewage sludge is improperly composted or treated to inactivate pathogenic micro-organisms, waiting time between application and planting of ready-to-eat crops is not respected and produce is harvested too soon after application or even as a result of the (prolonged) survival of organisms in the soil (De Roever, 1998; Natvig et al., 2002). *Salmonella* and *E. coli* can survive for several weeks (up to 21 weeks) in manure-fertilized soils. Depending on the season, when manure was incorporated in the soil, less decrease of both enteric bacteria in the soil and more contamination of the vegetables (such as arugula and radishes) was observed (Natvig et al., 2002). Similarly, *E. coli* O157:H7 could persist for more than five months in soil after application of contaminated compost or irrigation water (Islam et al., 2004).

Wild and domestic animals, e.g. birds and mammals, including rodents, are also a source of pathogenic bacteria in the agricultural environment by direct contamination (e.g. droppings) of the crop and contamination of the irrigation water (De Roever, 1998). Droppings of a deer were identified as the contamination source of strawberries with *E. coli* O157:H7 (Laidler et al., 2013). In addition, evidence was found for the transmission of pathogenic *E. coli* via insects, e.g. the fruit fly (*Ceratitis capitata*) (Janisiewicz et al., 1999), the vinegar fly (*Drosophila melanogaster*) (Sela et al., 2005) and the house fly (*musca domestica*) (Iwasa et al., 1999; Wasala et al., 2013).

The lack of good hygienic practice by the farm workers can lead to cross-contamination, especially in transmitting enteric viruses as norovirus and hepatitis A (Brackett, 1999; De Roever, 1998). Furthermore, cross-contamination of harvesting or processing equipment and human handling beyond the harvest stage may play a role as vehicle of contamination (Gil et al., 2015).

1.1.3 Survival of enteric pathogens on fresh produce

In the production field, the phyllosphere is characterized by a number of extreme and often fluctuating environmental conditions, e.g. temperatures, osmotic conditions, heavy rainfall and solar UV irradiation. Bacteria must either overcome these hurdles on the plant surface or find a way to enter plant tissue. The rugged topography of the plant landscape and the spatial heterogeneity in physicochemical conditions may create microsites for the survival of enteric pathogens (Berger et al., 2010; Brandl, 2006). To establish themselves on the plant surface, human pathogens attach to the plant surface using various adhesion methods, whereby fimbriae (curli), lipopolysaccharides and exopolysaccharides or flagella play an important role in the interaction between bacteria and biological surfaces (Olaimat & Holley, 2012; Seo & Matthews, 2012). Shaw et al. (2008) showed that EspA filaments allowed *E. coli* O157:H7 to attach to spinach and lettuce leaves. In Barak et al. (2005), *Salmonella enterica* genes (important for the virulence in animals, e.g. *RpoS* and *AgfD* gene) were found to be required for the colonization on plants. In addition, a biofilm can be developed and occurs as groups of bacterial cells aggregate in exopolysaccharide materials that can protect the cells from environmental stresses, including desiccation, UV and bacterial agents (Morris & Monier, 2003). The most common areas of bacterial aggregations on plants were at the base

of trichomes, around the stomata and along veins in the leafs. These structures promote water availability and nutrient leaching that in turn support microbial growth (Olaimat & Holley, 2012). Once fruit and vegetables were contaminated, it was observed that enteric pathogens (such as *Salmonella* and *E. coli*) were able to grow or survive for prolonged periods of time (Lynch et al., 2009). Low water activity is considered as one of the main limitations of bacterial growth on plant surfaces (Brandl, 2006). Survival of microorganisms is affected by the nutrient availability, toxic compounds released by the plant and competition from other microorganisms (Olaimat & Holley, 2012). *E. coli* O157:H7 could be detected for 77 and 177 days on lettuce and parsley, respectively, grown in manure-amended soils under field conditions (Islam et al., 2004). *Salmonella* was still detectable on basil leaves 100 days following spray irrigation with 8.5 log cfu/ml (Kisluk et al., 2013). Zhang et al. (2009) reported that *E. coli* O157:H7 could survive for at least 25 days on lettuce leaves, with a greater survival on the abaxial side of the leaves than on the adaxial side.

After harvesting and processing, fresh produce is packed and stored in several ways (e.g. washed vs. non-washed, whole vs. fresh-cut, open vs. closed packaging). The survival and multiplication of foodborne pathogens on fresh produce during storage is influenced by several factors which may be classified as intrinsic or associated with food material and extrinsic or associated with the environment surrounding the food, e.g. temperature, time, pH, water activity and relative humidity, packaging, type of produce and bacterial strain (Francis & O'Beirne, 2001; Likotrafiti et al., 2013; Stine et al., 2005). High humidity and short-term non-refrigerated storage can support the growth of pathogenic bacteria such as *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* on leafy greens and herbs. Temperature is the most important factor associated with bacterial growth and survival and therefore a critical control factor for food safety (FAO/WHO, 2008). Together with the storage temperature, the pH determines whether there is growth of bacteria on fresh fruit, whereby acidic fruit does not allow growth of pathogens (De Roever, 1998).

1.2 Fresh herbs and soft fruits

Fresh herbs are increasingly gaining popularity in the kitchen in Western European countries, such as Belgium due to changing eating habits and increased use of fresh herbs in ready-to-eat (RTE) meals. The majority of fresh herbs is consumed raw or added to food after cooking as decoration or as seasoning because of their distinctive flavors, colors and aromas. Herbs can be bought in retail stores as a whole plant, but nowadays also as conveniently pre-packed cut leaves. Most pre-cut herbs are not grown in substrate in controlled environments but in soil and in open field with either drip or overhead irrigation. Therefore, they may be susceptible to microbial contamination, as other leafy greens (CBI, 2010; Elviss et al., 2009; Zweifel & Stephan, 2012). Due to the seasonal production in Belgium and many other European countries, sourcing and import of fresh herbs from other regions is needed to meet the year-round demand.

Screenings of imported herbs resulted in the detection of enteric pathogens, mainly originating from South-East Asia. In 2005, 32 out of 244 imported herbs in London from non-EU countries were contaminated with *Salmonella*, including four varieties of basil grown in Thailand. In 2006, 5 out of 298 fresh herbs analyzed in the UK were contaminated with *Salmonella*, including coriander, curry leaves and holy basil grown in India or Thailand (Elviss et al., 2009; Surman-Lee et al., 2008). In Norway in 2005 and 2007, 28% and 15% respectively, of imported fresh herbs (basil, mint and coriander) from South-East Asia were contaminated with *Salmonella* (NSCFS, 2008). Fresh herbs, such as coriander and basil, are not always imported from South-East Asian countries, but can also be sourced from Mediterranean countries, such as Israel and Cyprus, or in the summer time they could be of local (Belgian) production. Little information is available on the sanitary quality and pathogen contamination of the herbs coming from these cultivation regions. In the UK, *Salmonella* Senftenberg has been detected on pre-packed basil which was grown in Israel and caused foodborne illnesses in England and Wales (Pezzoli et al., 2008). Also in Denmark (2006) a foodborne outbreak of Enterotoxigenic *E. coli* (ETEC) and *Salmonella* Anatum was associated with basil imported from Israel (Pakalniskiene et al., 2009). However, no other screening results have been published, leaving a data gap for further exposure and risk assessment. For leafy greens (to be used as salads), *E. coli* was identified as suitable for a

Hygiene Criterion at primary production. *E. coli* could be considered for validation and verification of Good Agricultural/Hygiene Practices (EFSA, 2014b).

Sweet basil (*Ocimum basilicum*) belongs to the botanically diverse group of aromatic plants and is one of the oldest herbs belonging to the *Ocimum* genus and the Lamiaceae (Labiatae) family. Basil is characterized by the presence of high numbers of background microbiota and contains essential oils, such as eugenol, linalool and methyl chavicol, with an antibacterial, antifungal, antiviral and insecticidal activity (Elgayyar et al., 2001; Suppakul et al., 2003; Ulukanli & Karadag, 2010; Wojcik-Stopczynska et al., 2010). For many years, the essential oils have been extensively used in the flavoring of confectionary and baked goods, sausages and meats, salad dressings, etc. and have also found a wide application in perfumery and dental and oral products (Suppakul et al., 2003). Indigenous microbiota may compete with pathogens for physical space and specific nutrients or produce antagonistic compounds that negatively affect the viability of the pathogens on fresh produce (Francis & O'Beirne, 1998; Liao & Fett, 2001). However, little is known about the persistence of foodborne pathogens on basil plants and harvested leaves that produce antimicrobial compounds. The emerging outbreaks raise concern as basil produces various antimicrobial compounds. Kisluk et al. (2013) suggest that the emerging outbreaks may reflect ecological changes that occurred as a result of development of resistance to essential oils. The *Salmonella* Senftenberg strain, a clinical isolate of such an outbreak (Pezzoli et al., 2008), survived better on basil leaves than *S.* Typhimurium and showed lower susceptibility to essential basil oils.

Soft fruits, such as strawberries (*Fragaria*), are non-climacteric, highly perishable fruits, which are susceptible to mechanical injuries, physiological deterioration, water loss and microbiological decay (Siro et al., 2006). Strawberries present many specific nutritional characteristics known to have health benefits. They are particularly rich in vitamin C, contain a high amount of potassium and a high content of ellagic acid. The latter phenolic compound is known as a naturally dietary antimutagen and anticarcinogen (Davis et al., 2007; Maas et al., 1991). Strawberry plants can be grown in soil or soilless cultures in protected environments or in open fields. Berries are harvested throughout the fruiting season and usually manually picked and directly placed in their final packaging for sale to the consumer. They are considered as high risk fruits as they are harvested manually, not washed before packaging and often consumed raw. However, due to the low pH (3.2 to 4.2) no growth or

survival of pathogens occurs (Knudsen et al., 2001). Nevertheless, it has been shown that *Salmonella* and *E. coli* O157:H7 can survive or even grow on the surface or on cut or bruised acidic produce such as apples, tomatoes and strawberries (Asplund & E., 1991; Dingman, 2000; Knudsen et al., 2001; Yu et al., 2001). An outbreak with *E. coli* O157:H7 in the US in 2011 with 15 cases, of which 2 were fatal, was caused by strawberries that were contaminated on the field by wildlife contact, namely deer feces (Laidler et al., 2013).

Strawberries are an important product in Belgium with regard to production volumes and sales. In 2012, almost 51,000 ton strawberries were traded on the Belgian auctions of which 40,500 ton was produced in Belgium. Seventy to eighty percent of the Belgian strawberry production is exported, mainly to other European countries, making strawberries the second most important product, after tomatoes, in sales of the auctions of the Union of Belgian Horticultural cooperatives (VILT, 2013; VLAM, 2014). Since there is no routine or regular monitoring of strawberries in Europe, limited information about the prevalence of enteric pathogens and the levels of generic *E. coli* on berries is available (EFSA, 2014b). Currently available studies suggest low prevalence of the pathogens *Salmonella* and STEC (*E. coli* O157) on strawberries (0/173 (Johannessen et al., 2002), 0/11 (Mukherjee et al., 2004), 0/194 (Mukherjee et al., 2006), 0/31 (Boraychuk et al., 2009) and 0/36 (Yoon et al., 2010)). Due to this lack of microbial data, with regard to enteric pathogens and fecal indicator bacteria such as *E. coli,* on berries, it is currently not possible to assess the suitability of an *E. coli* Hygiene Criterion at the primary production (EFSA, 2014b).

Good agricultural practices (GAP) and good hygiene practices (GHP) should be the primary objective of operators producing berries and leafy green vegetables (EFSA, 2014b, 2014c). Each production environment (including open field and enclosed or greenhouse production) should be evaluated independently for hazards as it represents an unique combination of numerous characteristics that can influence occurrence and persistence of pathogens in or near fields for growing berries or fresh herbs. In particular, evaluation of the production area may identify potential sources of fecal contamination.

1.3 Enteric pathogens: *Salmonella* and STEC

1.3.1 *Salmonella*

Salmonella, belonging to the family of the Enterobacteriaceae, are motile, Gram-negative, oxidase negative, facultative anaerobe rods. The genus is composed of three species, *Salmonella enterica, Salmonella bongori* and *Salmonella subterranea*, and *S. enterica* is subdivided in six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*. The subspecies can be further divided in serotypes. Serotypes typically involved in salmonellosis of mammals (including humans) almost invariably belong to the subspecies *enterica* (Heyndrickx et al., 2005; Shelobolina et al., 2004). More than 2600 serotypes of zoonotic *Salmonella* exist, although a limited number are associated with most human infections and the prevalence of different serotypes may change over time. In the EU (2012), the serotypes Enteritidis and Typhimurium (+ monophasic 1,4,[5], 12:i:-) are most frequently associated with human illness (Figure 4). *S.* Enteritidis is mostly associated with consumption of eggs and poultry meat, while *S.* Typhimurium with consumption of pig and bovine meat (EFSA, 2014a). Furthermore, strains are categorized as typhoidal (bloodstream infection) and non-typhoidal, depending on the disease syndrome they cause. Typhoidal *Salmonella* cause typhoid fever and are only related to the serotypes Typhi and Paratyphi. Strains of non-typhoidal *Salmonella* mostly cause intestinal infections (gastroenteritis) accompanied with diarrhea, fever, nausea and abdominal cramps that last a few days. Symptoms are sometimes mild and most infections are self-limiting. However, in some patients, the infection may be more serious and the associated dehydration can be life-threatening (EFSA, 2014a). Less commonly, non-typhoidal *Salmonella* can cause extra-intestinal infections such as urinary tract infection. Human illness is mostly foodborne, but *Salmonella* can also be transmitted by direct contact with animals, by water and, occasionally, by human contact (Nataro et al., 2011). The main reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals (EFSA, 2014a). Depending on the virulence of the *Salmonella* strain, a certain dose-response model, which describes the relationship between the ingested dose and the risk of infection, was observed. It was concluded that a wide variation in response occurred between species of *Salmonella*, but also from strain to strain of the same species (Teunis et al., 1996) .

Salmonella is the second most common zoonosis in humans in the EU (1,531 reported foodborne outbreaks in 2012). In 2012, a total of 91,034 confirmed cases of salmonellosis were reported by 27 EU Member States. However, a significantly decreasing trend of salmonellosis was observed during several years and continued in 2012 (4.7% decrease compared to 2011). The decreasing trend is likely to be mainly related to the successful *Salmonella* control programs in fowl (*Gallus gallus*) populations that are in place in the EU (EFSA, 2014a). Moreover, a clear seasonal trend of confirmed salmonellosis was reported, with the most confirmed cases during the summer months. Ten Member States of the EU provided information on hospitalization for some or all of their cases (10.1% of all confirmed cases). On average, 45.1% of the confirmed salmonellosis cases were hospitalized. Moreover, fourteen Member States provided information about the outcome of their cases and of these eight member states reported a total of 61 fatal cases (non-typhoidal salmonellosis) (fatality rate = 0.14%) (EFSA, 2014a).

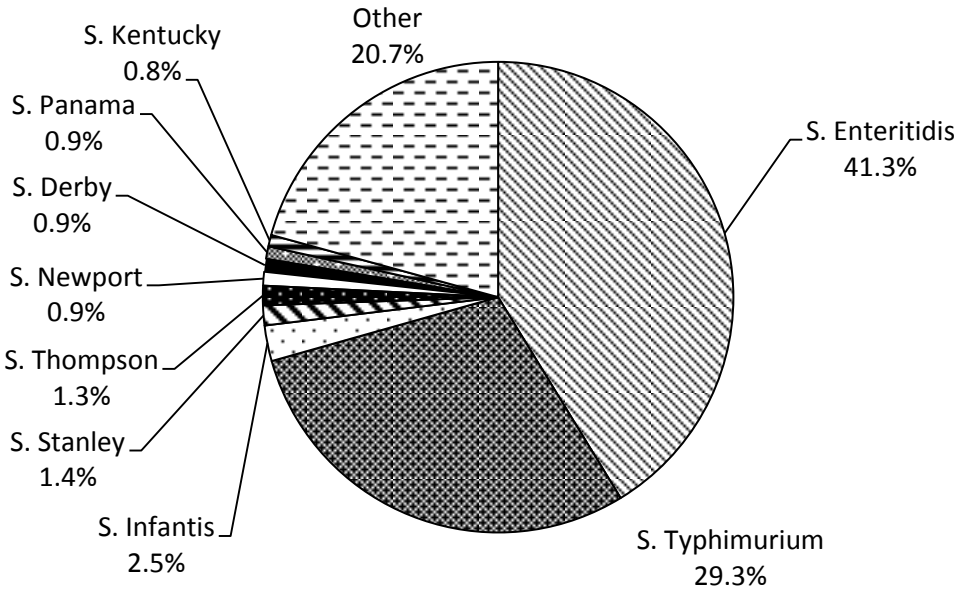

Figure 4: Overview of the most common *Salmonella* serotypes detected in humans in the EU/EEA in 2012 (N = 82,409) (EFSA, 2014a)

Salmonella infections were mostly associated with FoAO, primarily broiler meat, pig meat and bovine meat, but also with eggs and egg products, milk and dairy products, fruit and vegetables and fish and other fishery products. Focusing on fresh produce, further investigation by the Member States revealed that *Salmonella* was detected in 9 of the 20 reported investigations on vegetables. The highest prevalence was found in a Danish

investigation of leafy greens originating from EU countries (3 out of 33 samples tested positive). In 8 of the 18 reported investigations of *Salmonella* in spices and herbs, one or more samples were found to be *Salmonella* positive. Again the highest proportions was found in a Danish investigation of imported fresh herbs and spices (9.4% of 60 batches) (EFSA, 2014a).

1.3.2 STEC

E. coli, belonging to the family of the Enterobacteriaceae, are Gram-negative, oxidase negative rods and occur naturally in the lower part of the intestines of humans and warm-blooded animals. *E. coli* are facultative anaerobe and can either be motile or non-motile. *E. coli* is known as a harmless commensal of the gastrointestinal tract, however, there are specific pathogenic groups which are capable of causing disease in humans, both extra-intestinal (ExPEC) and enteric/diarrheagenic illness. ExPEC infections are primarily urinary tract infections and sepsis/meningitis (Croxen et al., 2013; Nataro et al., 2011). The six major diarrheagenic *E. coli* pathotypes are Shiga toxin-producing *E. coli* (STEC), Enteropathogenic *E. coli* (EPEC), Enterotoxinogenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC) and Diffusely adherent *E. coli* (DAEC). Isolates belonging to STEC pathotypes and one Enteroaggregative Hemorrhagic (EAHEC) pathotype O104:H4 are of particular importance in the context of food safety (EFSA, 2013c). Different serotypes may belong to more than one pathotype group. STEC is defined as an *E. coli* strain possessing Shiga toxin genes (*stx1* and/or *stx2*), typically acquired by a lambdoid bacteriophage. STEC can cause diarrheal illness (accompanied by fever, abdominal cramping or vomiting) with some cases leading to hemorrhagic colitis (HC; bloody diarrhea), potentially fatal hemolytic uremic syndrome (HUS) and thrombocytopenia (low blood platelet count). HUS is characterized by acute renal failure, anemia and lowered platelet counts. HUS develops in up to 10% of the patients infected with STEC O157 and is the leading cause of acute renal failure in young children (EFSA, 2014a). Enterohemorrhagic *E. coli* (EHEC) is a subset of STEC and is described by its association to HC and HUS. In addition to the *stx* genes, EHEC generally possess the *eae* gene (on the locus of enterocyte effacement (LEE)), required for attachment and effacing (A/E) lesions. However, EHEC has also been used in the literature to describe LEE-negative STEC strains (Croxen et al., 2013; EFSA, 2013c; Griffin et al., 1988; Remis et al., 1984; Riley et al., 1983). Numerous outbreaks have been attributed to STEC O157, but it is

recognized that non-O157 STEC, in particular O26, O103, O121, O111 and O145, can cause outbreaks and HUS (Bettelheim, 2007; Brooks et al., 2005; EFSA, 2013c; Guth et al., 2005; Sonntag et al., 2004; Tozzi et al., 2003). Of these non-O157 STEC, STEC O26 was reported to cause most STEC illnesses in Europe between 2007 and 2011, excluding the outbreak of STEC O104 (Germany 2011) (EFSA, 2013c). Ruminants, such as cattle, goats and sheep, are widely known to be the major reservoir for pathogenic STEC and their fecal matter represents an important source for human illness. STEC can be transmitted through ingestion of contaminated food and water, person-to-person contact, contact with animals or contact with environments or dust contaminated with fecal material (EFSA, 2013c). The dose-response for diarrheagenic *E. coli* varies by strain and pathotype. The STEC O157 outbreak-strain was found to be highly infectious, whereas for EPEC a lower infectivity was observed (Teunis et al., 2004).

In 2012, 5,671 confirmed STEC cases were reported in the EU, a decrease of 40% taking the large outbreak of STEC O104:H4 in Germany, associated with sprouted seeds, into account. On average, 36.5% of the confirmed cases were hospitalized and 12 deaths due to STEC infection were reported in 2012 (fatality rate of 0.36%). There was an significantly increasing EU trend of confirmed human STEC infections in 2008-2012 (even without the 2011 outbreak). The increase is mostly likely due to a generally increased awareness of the disease and increased detection (e.g. all STEC instead of STEC O157 only) and reporting by countries, especially due to the 2011 outbreak. A clear seasonal trend in the confirmed STEC cases was reported in the EU during 2008-2012 with more cases reported in the summer months. The most commonly reported serotype was O157, followed by O26 (Figure 5) (EFSA, 2014a).

Karmali et al. (2003) developed a classification for STEC seropathotypes (A through E) according to the occurrence of serotypes in human illness, outbreaks and/or HUS (Table 2). Seropathotype A represents high risk, while seropathotype D and E minimal risk. However, the classification does not define pathogenic STEC and provides no exhaustive list of pathogenic serotypes (EFSA, 2013c). Foods of bovine or ovine origin are frequently reported as source of human STEC infections, whereby contaminated bovine meat is considered as the major source of foodborne STEC infections. Other important food sources include fecally contaminated vegetables and drinking water (EFSA, 2014a).

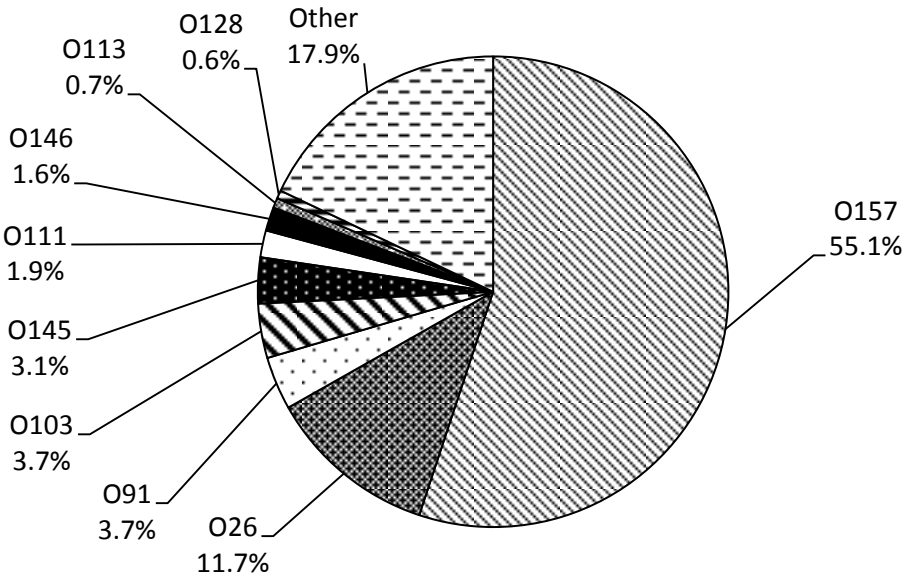

Figure 5: Overview of the most common STEC serotypes detected in humans in the EU/EEA in 2012 (N = 3,558) (EFSA, 2014a)

Table 2: Classification of STEC serotypes into seropathotypes by Karmali et al. (2003)

Seropatho-type	Relative incidence	Frequency of involvement in outbreaks	Association with severe illness (HUS and/or HC)	Serotypes
A	High	Common	Yes	O157:H7, O157:NM
B	Moderate	Uncommon	Yes	O26:H11, O103:H2, O111:NM, O121:H19, O145:NM
C	Low	Rare	Yes	O91:H21, O104:H21, O113:H21, O5:NM, O121:NM, O165:H25
D	Low	Rare	No	O7:H4, O69:H11, O103:H25, O113:H4, O117:H7, O119:H25, O132:NM, O146:H21, O171:H2, O172:NM, O174:H8, Orough:H2.
E	Non-human only	NA	NA	O6:H34, O8:H19, O39:H49, O46:H38, O76:H7, O84:NM, O88:H25, O98:H25, O113:NM, O136:H12, O136:NM, O153:H31, O156:NM, O163:NM.

NA = not applicable; NM = non-motile

1.4 Detection of enteric pathogens on fresh produce

Methods for detection of *Salmonella* in FoNAO are well developed and analytical reference methods standardized and widely adopted across laboratories testing food. For Official Control: ISO standard method 6579[1] is prescribed in Regulation 2073/2005[2] when analyzing pre-cut RTE fruit and vegetables in the scope of the verification of compliance with the currently established food safety microbiological criterion for *Salmonella*. Alternative methods based on modifications of the ISO method using alternative enrichment media or isolation media (chromogenic media) or using immunoassays and real-time PCR are also available for rapid detection of *Salmonella,* and many of these methods have been ISO 16140[3] validated showing performance characteristics equivalent to the ISO standard method 6579 (EFSA, 2014c). Delibato et al. (2014) reported the validation of a non-patented real-time PCR method for detection of *Salmonella* in pork meat, developed by Josefsen et al. (2007), according to the ISO 16140:2003. It was concluded that the real-time PCR method represented an excellent alternative to the ISO standard method.

The standard detection method for *E. coli* O157 has been available since 2001 (ISO 16654:2001[4]). In 2012, ISO 13136:2012[5] has been published. Still this latter method and, in particular, its implementation in the routine lab is challenging. This because, no standard medium is available and it is advised to pick up 50 presumptive colonies from a suitable medium (e.g. TBX) for further confirmation. Moreover, the detection of pathogenic *E. coli* is more debated. The European Food Safety Authority (EFSA) stated that it is still not possible to fully define human pathogenic STEC or identify factors for STEC that absolutely predict the

[1] ISO 6579:2002. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. International Organization for Standardization, Geneva, Switzerland. (First edition dating back to 1981)

[2] Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. OJ L 338, 22.12.2005, p.1-26

[3] ISO 16140:2003: Microbiology of food and animal feeding stuffs -- Protocol for the validation of alternative methods. International Organization for Standardization, Geneva, Switzerland.

[4] ISO 16654:2001: Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of *Escherichia coli* O157. International Organization for Standardization, Geneva, Switzerland.

[5] ISO 13136:2012: Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* STEC) and the determination of O157, O111, O26, O103 and O145 serogroups. International Organization for Standardization, Geneva, Switzerland.

potential to cause human illness. It seems to be prudent to consider the presence of *stx* genes in an isolated *E. coli* strain as a hazard able to cause human illness (EFSA, 2013c).

A molecular classification, using genes encoding virulence characteristics additional to the presence of *stx* genes, may give a first common approach. This could assist the competent authorities of the EU Member States in conducting a risk assessment when confronted with positive STEC results and in taking the appropriate measures to ensure that the risk for the consumers is reduced as much as possible. For example, strains positive for *stx2* and *eae* or *aggR* (plasmid encoded regulator) genes are associated with higher risk of more severe illness than other virulence gene combinations. A contaminated RTE product with an isolate of one of the STEC serotypes O157, O26, O103, O145, O111 and O104 in combination with *stx* and *eae* or *aaiC* and *aggR* genes should be considered as presenting a potentially high risk for diarrhea and HUS. Other serotypes in combination with these genes are regarded as a potentially high risk for diarrhea, but currently unknown for HUS. In addition, the O104:H4 outbreak in Germany, demonstrated the difficulties of detecting the 'new' pathogenic STEC strains by screening only for the *eae* gene or focusing on a restricted panel of serotypes (EFSA, 2013b, 2013c).

1.4.1 *Salmonella*

The classical detection method for *Salmonella* in food (ISO 6579:2002), based on cultivation and biochemical confirmation, is well established (Figure 6). Samples are enriched in a non-selective medium (Buffered Peptone Water (BPW)), followed by selective enrichment in two broths (Rappaport-Vassiliadis with Soya (RVS) and Muller-Kauffmann Tetrathionate-novobiocin (MKTTn)) and plating on two selective agar media including Xylose-Lysine-Deoxycholate agar (XLD). Presumptive colonies on the selective agar media should be further confirmed (biochemical and serological confirmation). In 2007, an amendment of ISO 6579 (ISO 6579:2002 FDAM1[6]) was published and recommended for the detection of *Salmonella* spp. in animal feces and in samples from the primary production stage. Instead of RVS and MKTTn broth, the modified semi-solid Rappaport-Vassiliadis (MSRV) agar plates were used for selective enrichment (Figure 6).

[6] ISO 6579:2002 FDAM 1: Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. AMENDMENT 1: Annex D: Detection of *Salmonella* spp. in animal faeces and in samples from the primary production stage

However, the method is labor intensive and results are only obtained after more than 4 days, in contrast to more rapid alternative methods, such as PCR, where the result is obtained after 1 to 2 days. Moreover, food matrices with a high level of background microbiota or an adverse environment for *Salmonella* can disturb the growth of *Salmonella*, e.g. overgrowth of low numbers of *Salmonella* by Enterobacteriaceae present on the food matrix during enrichment or a low pH which doesn't allow growth of *Salmonella* (Chajecka-Wierzchowska et al., 2012). Shearer et al. (2001) reported the necessity of using both selective enrichments and both selective media to detect *Salmonella* in fruit and vegetables, i.e. a single combination of selective enrichment and plating medium didn't allow detection of *Salmonella* in all cases. In addition, the media are highly selective causing inhibition of growth of some *Salmonella* strains.

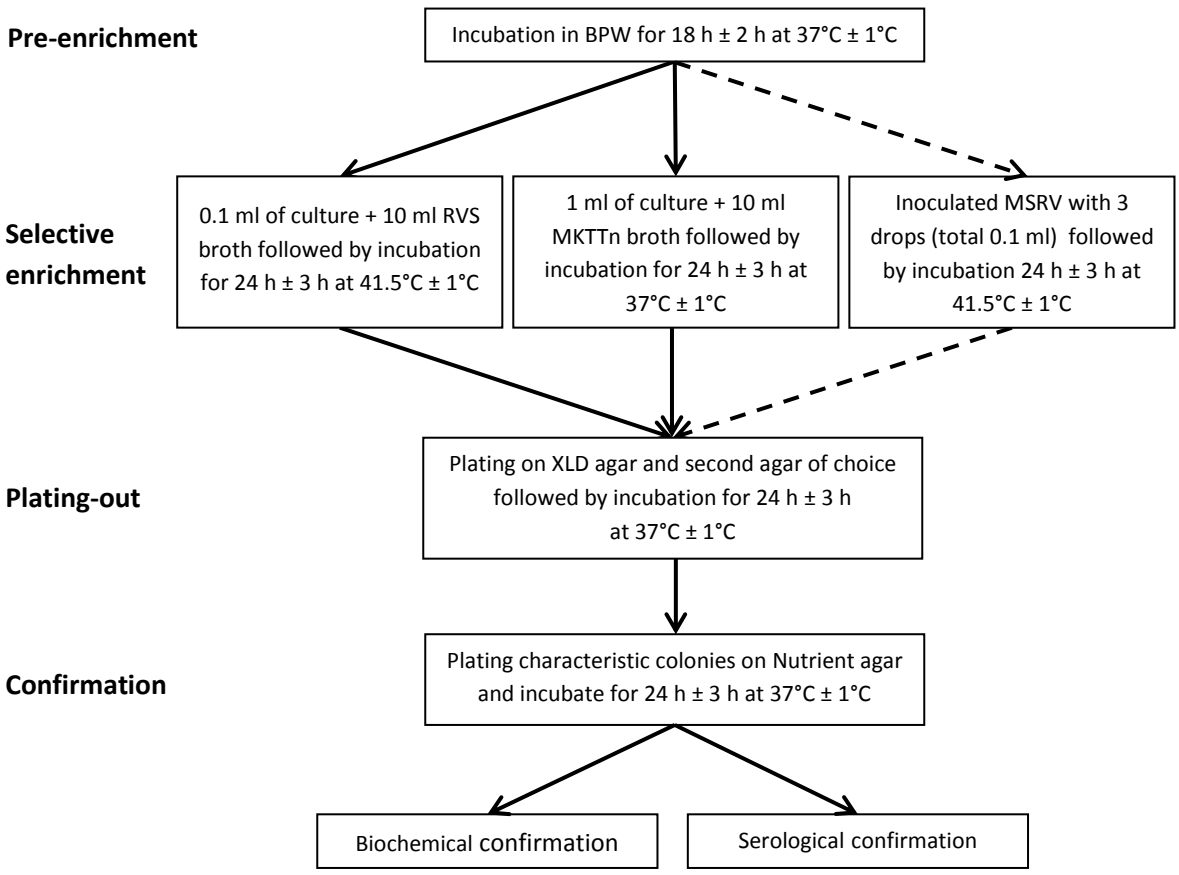

Figure 6: Overview of different steps of the detection method for *Salmonella* described in ISO 6579:2002 ( ) and ISO 6579:2002 FDAM1 ( )

1.4.2 STEC

Food testing methodologies for STEC were generally focused on (non-sorbitol-fermenting) STEC O157 which has several unique characteristics, that facilitate the detection, isolation and identification of this specific STEC serotype (Jinneman et al., 2012). ISO 16654:2001, the standard method for the detection of *E. coli* O157, proceeds as follows: (i) selective enrichment in modified Tryptone Soya Broth with novobin (mTSB+N) followed by, (ii) immunomagnetic separation (IMS) using beads coated with antibodies to bind *E. coli* carrying the O157 antigen and (iii) plating on Cefixime Tellurite Sorbitol MacConkey agar (CT-SMAC) and another selective medium. Presumptive colonies should be confirmed by biochemical and serological identification. Selective media are needed to favor isolation of *E. coli* O157 opposed to other Gram-negative bacteria in the sample. An incubation temperature of 41-42°C further enhances selectivity. For the isolation of stressed cells (heat-, freezing-, acid- or salt-stress), pre-enrichment in a non-selective broth is necessary to detect the cells, as STEC is highly infectious (see dose-response model described in Teunis et al. (2004)) and injured cells mostly retain their pathogenic properties (De Boer & Heuvelink, 2000). CT-SMAC is the most widely used plating medium for the isolation of classical STEC O157. As these strains do not ferment sorbitol and are β-glucuronidase (GUD) negative, transparent and almost colorless colonies with a pale yellowish-brown appearance are formed on CT-SMAC. A low concentration of cefixime is added to inhibit *Proteus*, but not *E. coli*, whereas tellurite is added to inhibit *E. coli* strains other than STEC O157. However, as some STEC strains are sensitive to tellurite and/or ferment sorbitol and are GUD-positive, the use of a second isolation medium is recommended. IMS increases sensitivity by concentrating *E. coli* O157 relative to background bacteria, which may grow on selective agars for *E. coli* O157 (De Boer & Heuvelink, 2000). Several studies have reported drawbacks of the current culture detection methods for STEC, e.g. selective enrichment (to avoid the growth of background microbiota which can outcompete the target organism) making it impossible to detect physically stressed cells (Hussein & Bollinger, 2008; Jasson et al., 2009) and overgrowth of isolation media by other microbiota (Jinneman et al., 2012; Tzschoppe et al., 2012; Weagant et al., 2011). Furthermore, non-O157 STEC phenotypically resemble commensal *E. coli* and are more difficult to detect than non-sorbitol-fermenting, β-GUD-negative STEC O157 (Bettelheim, 2003; Kalchayanand et al., 2012; Posse et al., 2008). No

standard differential agar media are available for non-O157 STEC. However, Posse et al. (2008) described a new selective differential agar medium for STEC serotypes O26, O103, O111 and O145, based on a mixture of carbohydrate sources (sucrose and sorbose), the β-D-galactosidase activity by adding isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and selective components, which allows colour-based distinction of these serotypes (Table 3). Combining the β-D- galactosidase-activity and the carbohydrate fermentation in a single medium allows differentiation of selected STEC serotypes from commensal strains. Furthermore, suspected phenotypes on the differential media for non-O157 STEC are plated on one or more confirmation media containing specific carbohydrates (D-arabinose, L-rhamnose, dulcitol and/or D-raffinose).

Table 3: Overview of substrate fermentation and colours of STEC O26, O103, O111 and O145 colonies on the differential medium for non-O157 STEC developed by Posse et al. (2008).

Bacteria	Substrate fermentation	Colours on differential media
STEC O26	Sucrose and sorbose	Bright red to dark purple
STEC O103	Sucrose	Blue-purple
STEC O111	Sucrose, D-arabinose and Dulcitol	Blue-purple
STEC O145	D-arabinose	Green

CHROMagar STEC (CHROMagar Microbiology, France) was developed to isolate O157 and non-O157 STEC, but no differentiation between the different serotypes can be made. CHROMagar STEC was found to be more selective than CT-SMAC in case of vegetable screening as sorbitol fermenting Enterobacteriaceae are present in high numbers on vegetables. However, it should be noted that some strains were not able to grow on CHROMagar STEC, e.g. sorbitol fermenting *E. coli* O157:H7, *E. coli* O26:H11 and *E. coli* O103:H2 (Hirvonen et al., 2012; Tzschoppe et al., 2012).

The occurrence of phenotypic variants of *E. coli* O157 and of other STEC serotypes necessitates testing for virulence characteristics, such as the presence of *stx* genes, enterohemolysin genes and the *E. coli* attaching and effacing (A/E) *eae* gene. The use of multiplex real-time PCR may prove to be a more convenient and rapid alternative for screening of these enteric pathogens in fruit and vegetables. Recent efforts have focused on specific STEC serotypes (e.g. O26, O111, O103, O121 and O145) and/or additional virulence characteristics. The key virulence factors in human STEC infections are the Shiga toxins (*stx1*

and *stx2* genes) and intimin (*eae* gene) (Karmali, 2004; Posse et al., 2007; Schmidt et al., 1999). Recently an ISO method for STEC (serogroups O157, O111, O26, O103 and O145) was published which uses a real-time PCR screening for virulence factors *stx1*, *stx2* and *eae* followed by serogroup detection in case of a *stx* and *eae* PCR positive enriched sample. In addition, STEC strains in *stx* positive (and *eae* positive or negative) samples have to be isolated (ISO 13136:2012). The GeneDisc PCR system, which allows simultaneous detection of multiple target genes (e.g. *stx1*, *stx2*, *eae*, $rfbE_{O157,}$ etc.), for the detection of STEC O26, O103, O111, O145 and O157 was evaluated in Beutin et al. (2009). A reliable detection was observed for the STEC associated virulence genes and genes encoded on somatic antigens of the major STEC serotypes. Jinneman et al. (2012) reported successful post-enrichment detection of STEC O104 and STEC O157 inoculated at 1 cfu/g on different sprouts with multiplex PCR screening (*stx1*, *stx2*, *uidA*, *wzy* O157 and *wzx* O104 genes). In contrast, Tzschoppe et al. (2012) reported that if low numbers (1-10 cfu/25 g) are present in the fresh produce samples of salad and sprouts, the numbers of STEC in broth after enrichment may be too low to be detected by PCR and isolation of the actual STEC strains by subsequent cultural methods may be difficult. Unlike food of animal origin, vegetables and fruit are characterized by the presence of high numbers of competing microbiota and inhibitory compounds. However, attention should still be paid to the potential occurrence of false positive and false negative results when using PCR. False positive results can be obtained by cryptic target genes (such as free *stx* phages) or by nonspecific amplification (Bettelheim, 2003). False negative results can be caused by PCR inhibition or DNA degradation through plant polysaccharides, phenolic compounds, DNases, etc., present in the enrichment broth (Wilson, 1997). Moreover, prior stress and injury or the presence of competing flora could impair or suppress the growth of the target bacteria during enrichment and thus lead to false negative PCR results (Verstraete et al., 2012). Another cause is the spontaneous loss of *stx* genes after multiple subcultivation steps or long preservation (Brooks et al., 1997; Feng et al., 2001; Karch et al., 1992), because these genes are encoded on temperate bacteriophages (Schmidt, 2001). Moreover, Joris et al. (2011b) noticed the loss of *stx* already during the first subcultivation step.

1.5 Risk assessment and risk ranking of enteric pathogens on fresh produce

The wide diversity of climatic and environmental conditions and farming practices (e.g. open field versus protected soilless cultivation (Figure 7) in fresh produce cultivation corresponds to various microbiological risk factors (e.g. water use for irrigation, access of domestic and wild animals, field history and adjacent land use) (EFSA, 2013b; Gil et al., 2015).

Figure 7: Different farming practices used in strawberry cultivation in Belgium: soil (A, B) versus soilless (e.g. peat) (C,D) and open field (A) versus protected (plastic tunnels or greenhouse) (B, C, D) cultivation.

As fresh fruit and vegetables receive minimal processing and are mostly consumed raw, contamination by pathogens can represent a serious risk for foodborne outbreaks. To assess, compare and evaluate the risk of foodborne illness from microbial and chemical hazards and the public health impact of an intervention, stakeholders in the system of food safety need

evidence-based, transparent and rigorous approaches. Moreover, in order to make policy, regulatory and other decisions, risks are often classified in some kind of priority ranking.

Quantitative microbial risk assessment (QMRA) aims to model the fate of pathogens and the associated health risk along the food chain. Moreover, the modelling allows to estimate the impact of an intervention or a combination of interventions in the food chain on the public health by scenario analysis 'what if' or insight in the 'most influencing factor' of a food chain by sensitivity analysis. The value of QMRA models is enhanced when combined with socio-economic analysis (e.g. cost-effectiveness and public support). QMRA models also foster the insight in the processes of the food pathway and highlight knowledge gaps, resulting in a better basis for objective, risk-based criteria and targets to promote risk-based decision, to improve risk communication and to focus data collection efforts (Havelaar et al., 2008).

Several QMRA models, relating to fresh leafy vegetables and bacterial pathogens, have been described in literature, e.g. for spinach associated with *E. coli* O157:H7 (Danyluk & Schaffner, 2011), leafy green vegetables in salad associated with *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (Franz et al., 2010), lettuce associated with *Listeria monocytogenes* (Ding et al., 2013) and *E. coli* O157:H7 (Ottoson et al., 2011). The development of QMRA models is however time consuming and needs a lot of data (e.g. prevalence of pathogens, growth/die-off during retail and home storage, consumer behavior, etc.). It may also contain several data gaps or assumptions (e.g. initial contamination level, dose-response modelling, etc.) leading to uncertainty in their outcomes.

Risk ranking is a useful alternative for QMRA models to set priorities in controlling or monitoring risks as input for risk management decisions instead of precise quantification of risk as objective in QMRAs. Several risk rankings for fresh produce have been conducted based on qualitative and/or quantitative evaluation of relevant criteria (Table 4). In EFSA opinion (2012), several tools were reported for risk ranking related to biological hazards, among others a risk ranking tool for fresh produce from the FDA (USA) (Anderson et al., 2011), the iRISK tool (Chen et al., 2013) and a swift quantitative microbiological risk assessment (sQMRA) (Evers & Chardon, 2010) tool aimed at comparing the risk of hazard-food product combinations. To set priorities in FoNAO, EFSA adapted the risk ranking tool reported by Anderson et al. (2011) (EFSA, 2013b). Moreover, a multicriteria-based ranking

relating to foodborne parasites, based on similar assessments conducted for zoonotic and infectious diseases (e.g. Anderson et al. (2011)), was performed during a joint FAO/WHO expert meeting (Table 4) (FAO/WHO, 2014).

Table 4: Overview of different criteria or components of risk ranking tools used to rank pathogen-fresh produce combinations.

FAO/WHO ranking of microbiological hazards (FAO/WHO, 2008)	FDA Ranking Tool[a] (Anderson et al., 2011)	FDA- iRisk[b] (Chen et al., 2013)	FAO/WHO ranking of foodborne parasites[a] (FAO/WHO, 2014)
Frequency and severity of disease	Epidemiological link	The food	Number of global food-borne illnesses
Size and scope of production	Disease multiplier	The hazard	Global distribution
Diversity and complexity of production chain/industry	Hospitalization and death rates	The population of consumers	Acute morbidity severity
Potential for amplification of foodborne pathogens through the food chain	Susceptible population	A process model (e.g. food production, processing and handling practices)	Chronic morbidity severity
Potential for control	Prevalence of contamination	Consumption patterns in the population	Fraction of illness that is chronic
Extent of international trade and economic impact	Relative infectivity	Dose-response relationships	Case-fatality ratio
	Consumption	Burden of disease measures associated with health effects (e.g. DALYs)	Increasing illness potential
	Shelf-life/growth potential		Trade relevance
			Impacts on economically vulnerable communities

[a]: For each criterion a score was assigned. Subsequently, scores were combined to an overall score whereby each criterion was weighted as a fraction of the total score. [b]: Web-based quantitative risk assessment which integrates data and assumptions from seven components.

In Europe, there is a growing interest in the safety of fresh produce since the EHEC outbreak in Germany (and other countries) in 2011. Subsequently to the risk ranking of FoNAO by EFSA which was published in January 2013 (EFSA, 2013b), more in depth studies for specific pathogen-commodity combinations were developed including an opinion on *Salmonella* and Norovirus in berries (EFSA, 2014b) and *Salmonella* and Norovirus in leafy greens (EFSA, 2014c) describing the whole production process and specific mitigation options to reduce contamination. Recently, also a QMRA study was published related to STEC and *Salmonella* in leafy greens eaten as salads (Pielaat et al., 2014) based on data collected in a large survey in the Netherlands (Wijnands et al., 2014). However, it is also of interest to know how the

potential risks of niche products such as basil and of general assumed safe fruits such as strawberries are relating to the risks presented by leafy greens. Hereby should factors, such as microbiological growth/survival, consumer handling and consumption patterns of basil and strawberries, which might be quite different from the overall well studied commodity of lettuce, be taken into account. As few data were available in Belgium (and Europe) on the presence of *Salmonella* and STEC on fresh basil and strawberries or in their production environment, this was also addressed in this PhD thesis.

CHAPTER 2: Multiplex real-time PCR and culture methods for detection of *Salmonella* Thompson and Shiga toxin-producing *Escherichia coli* in basil, strawberries and a lettuce mix

Redrafted from:

Delbeke, S., Ceuppens, S., Holvoet, K., Samuels, E., Sampers, I. & Uyttendaele, M. (2015). Multiplex real-time PCR and culture methods for detection of Shiga toxin-producing *Escherichia coli* and *Salmonella* Thompson in strawberries, a lettuce mix and basil. *International Journal of Food Microbiology,* 193: 1-7
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ABSTRACT

An appropriate approach of high throughput multi-screening was verified for *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC) in basil, strawberries and lettuce. Sample replicates were inoculated with *Salmonella* Thompson and STEC O157 or O26 (ca. 10-70, 100-700 and 1000-7000 cfu/25 g) and analyzed after one and five days of storage (strawberries and lettuce at 7°C and basil at 10°C). After 18-24 h of enrichment at 37°C in Buffered Peptone Water, detection was performed using the GeneDisc multiplex real-time PCR (*stx1*, *stx2*, *eae* and *iroB* genes) and selective culture media for isolation of STEC (with immunomagnetic separation (IMS)) and *Salmonella* in parallel. After one day, the pathogenic strains were recovered from all samples for all inoculum levels, whereas reduced detection rates of *S.* Thompson and STEC O157 were observed after five days of storage in case of strawberries, in particular for the lowest inoculums level, suggesting superior survival potential for STEC O26. Overall, this study indicates the ability of PCR based screening methods for reproducible multi-detection of low numbers (10-70 cfu/25g) of *Salmonella* and STEC in this type of foods. However, for the basil samples, PCR needed twofold dilution of the DNA extract to overcome inhibition. It was noted that on several occasions growth of competitive microbiota obstructed finding presumptive colonies on the selective agar media, whereas the use of an additional agar medium such as CHROMagar STEC (in this particular case without IMS) improved recovery rate of STEC.

2.1 Introduction

The present study aims to determine the suitability, with regard to the detection of a low number of cells on vegetables or fruits, of a PCR screening assay, namely GeneDisc multiplex real-time PCR (Pall GeneDisc Technologies, Bruz, France) and culture methods as described in ISO 16654:2001 and ISO 6579:2002 for high throughput multi-screening of STEC (in the present study exemplified by an O157 and an O26 STEC strain) and *Salmonella* (exemplified by a *Salmonella* Thompson strain) on strawberries, a lettuce mix and basil leaves.

2.2 Material and methods

2.2.1 Bacterial strains

Two STEC strains, serotypes O157 (*stx1*, *stx2* and *eae* genes positive) and O26 (*stx1* and *eae* genes positive), and one *Salmonella* strain (*iroB* gene present) were used for inoculation. Both STEC strains were clinical strains obtained from the collection of Prof Lieven De Zutter (Lab of Hygiene and Technology, Faculty of Veterinary Medicine, Ghent University). *Salmonella enterica* serovar Thompson RM1987N (STN) is a spontaneous nalidixic acid resistant mutant of *Salmonella* Thompson strain RM1987 and was received from Dr. Maria Brandl (ARS USDA, Albany, California, USA). Strain RM1987 is a previously described clinical isolate from a patient in a cilantro-linked outbreak in California (Brandl & Mandrell, 2002; Brandl et al., 2005).

To assess their typical characteristics, STEC O157, STEC O26 and *S.* Thompson were analyzed by the GeneDisc multiplex real-time PCR (PALL GeneDisc Technologies) and plated on selective or chromogenic media. STEC O157 was plated on Cefixime Tellurite Sorbitol MacConkey (CT-SMAC; Oxoid) agar to which 8 mg/l novobiocin (Sigma Aldrich, Steinheim, Germany), 0.05 g/l isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Aldrich) and 0.05 g/l X-galactopyranoside (X-gal, Promega, Madison, USA) was added (= refer to as adapted CT-SMAC (aCT-SMAC)). The pH was adjusted to 7.4 (De Boer & Heuvelink, 2000; Hussein & Bollinger, 2008; Posse et al., 2008). STEC O26 was plated on MacConkey agar (MAC; BD Biosciences) to which 6 g/l D-sucrose (Sigma Aldrich), 6 g/l L-Sorbose (Sigma Aldrich), 3.5 g/l bile salts No. 3 (Oxoid), 0.05 g/l X-gal, 0.05 g/l IPTG, 8 mg/l novobiocin and 2.5 mg/l tellurite

(Sigma Aldrich) was added as described in Posse et al. (2008) and to which will be referred to as adapted MAC (aMAC) in the present research. Both STEC strains were also plated on CHROMagar STEC (CHROMagar Microbiology, Paris, France) and Petrifilm Select *E. coli* (3M, Diegem, Belgium) and *Salmonella* on *Salmonella Brilliance* agar (SB, Oxoid) and Xylose-Lysine-Desoxycholate agar (XLD, Oxoid). The selected strains developed typical colonies regarding colour and morphology on their selective culture media, except STEC O157 which could not be detected on the Petrifilm since, as most STEC O157 strains, this strain was negative for glucuronidase (Osek, 2004; Ratnam et al., 1988; Thompson et al., 1990). The GeneDisc analysis resulted in: *S*. Thompson *iroB* positive, STEC O157 *stx1, stx2* and *eae* positive and STEC O26 *stx1* and *eae* positive.

2.2.2 Inoculation procedure

The strains, stored at -75°C on glass beads, were revived in 10 ml Tryptone Soya Broth (TSB, Oxoid) for 24 h at 37°C, followed by sub-culturing 100 µl of the 24 h culture in another 10 ml TSB (24 h; 37°C) and plating the subculture on Tryptone Soya agar (TSA, Oxoid) (24 h; 37°C) for enumeration. All strains grew to approximately 10^9 cfu/ml.

Strawberries, the lettuce mix (containing biondi, lollo, frisee and red butterhead lettuce) and basil were purchased from a local wholesaler in Ghent and stored at 4°C until inoculation on the same day. Samples of 25 g of the lettuce mix, strawberries and basil were transferred to plastic bags (18 x 18 cm; 50 EA plain 420 nm film, 1500 ml $O_2/m^2/d$), plastic punnets (± 254 cm^3) with holes (± 16 cm^2) in the lid and plastic bags (20 x 20 cm; 20 EA plain 420 nm film, 4600 ml $O_2/m^2/d$), respectively. Each commodity (left uncut) was inoculated with two different pathogen mixtures (either *E. coli* O157/*Salmonella* Thompson or *E. coli* O26/*Salmonella* Thompson) and with three inoculum levels (10-70; 100-700; 1000-7000 cfu/25 g) in triplicate. Therefore, the subculture of each strain was 10-fold diluted in Peptone Physiological Salt solution (PPS; 1.0 g/l neutralized bacteriological peptone (Oxoid), 8.5 g/l NaCl (Sigma Aldrich, Germany)) and 1 ml of STEC O157 or O26 and 1 ml of *S.* Thompson of the appropriate 10-fold dilution was inoculated (by means of droplets) on the lettuce mix or basil. Only 100 µl of the appropriate dilution of each pathogen was inoculated on the strawberry samples to avoid quality defects and the inoculum was put on the fruit and not on the calyx. Blank samples were used as negative controls. The bags of lettuce and

basil were closed by heat-sealing. After one and five days of storage at 7°C for lettuce and strawberries or 10°C for basil leaves (10°C to prevent cold damage and visual quality defects), 25 gram samples were taken for detection of *Salmonella* and STEC O157 or O26. Total *E. coli* was enumerated from samples with the highest inoculum level and blank samples using the Petrifilm Select *E. coli* (24 ± 2 h incubation at 37°C).

2.2.3 Detection procedure

The simultaneous detection of both *Salmonella* Thompson and STEC started with enrichment of 25 g samples in 225 ml Buffered Peptone Water (BPW, Oxoid) in a sterile filter blender bag, which were homogenized by a stomacher for 30 s and incubated at 37°C for 18 to 24 h. Both multiplex real-time PCR detection (GeneDisc Cycler) and culture based isolation were performed in parallel (Figure 8).

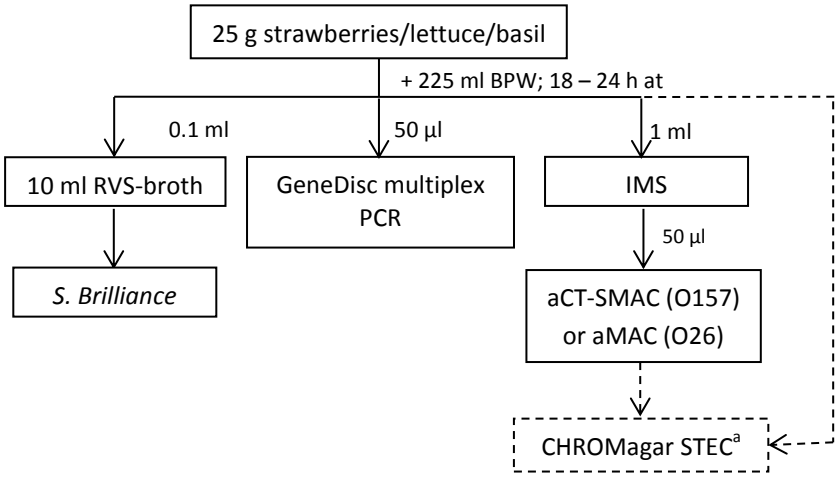

Figure 8: Overview of the detection procedure of *S.* Thompson and STEC (O157 or O26) inoculated on strawberries, a lettuce mix and basil ([a]: only performed when doubtful results were obtained on aCT-SMAC or aMAC).

2.2.3.1 Detection of *Salmonella* and STEC by GeneDisc multiplex real-time PCR

A custom-made GeneDisc plate was created by Pall GeneDisc Technologies for the simultaneous detection of *Salmonella* (the *iroB* gene) and the STEC virulence genes *stx1*, *stx2* and *eae*. DNA extraction and GeneDisc multiplex real-time PCR were performed according to the instructions of the manufacturer as previously described in Beutin et al. (2009). In short, 50 µl of the enriched BPW broth was transferred to a lyse tube (extraction pack FOOD 1,

PALL GeneDisc Technologies), incubated for 10 min at 100°C in a heating block and centrifuged for 2 min at 10,000 × *g*. Then, 36 µl of the DNA-extract and 36 µl of the Master Mix (Pall GeneDisc Technologies) were transferred to the GeneDisc plate, which was subsequently loaded in the GeneDisc Cycler. In the GeneDisc plate an internal amplification control was included. Inhibition of the PCR reaction could be observed on the screen of the GeneDisc Cycler by a delay (shift in the CT-value, > 33) or the absence of an inhibition curve. When inhibition occurred, the DNA extract was twofold diluted in a dilution buffer (extraction pack FOOD 1) and the PCR analysis was repeated.

2.2.3.2 Detection of *Salmonella* Thompson by culture method

The enriched BPW broth was selectively enriched in Rappaport Vassiliadis Soya (RVS, Oxoid) broth (preheated to room temperature) for 24 h ± 3 h at 42°C (ISO 6579:2002) followed by inoculating the selective medium SB by means of a sterile loopful and incubating at 37°C for 24 h ± 3 h. SB agar was chosen over XLD as better results were obtained concerning the inhibition of the growth of background microbiota during preliminary studies.

2.2.3.3 Detection of STEC O157 and O26 by culture methods (including IMS)

In parallel to multiplex PCR, samples were analyzed for STEC O157 and O26 by the use of immunomagnetic separation (IMS) followed by plating on aCT-SMAC and aMAC, respectively. IMS was performed starting from 1 ml enriched BPW broth and 20 µl Dynabeads anti-*E. coli* O157 (Invitrogen Dynal AS, Oslo, Norway) or Dynabeads EPEC/STEC O26 (Invitrogen Dynal AS) in 1.5 ml microcentrifuge tubes on a shaker (orbital shaker os 10 basic, IKA, Germany) for 30 min at 140 rpm at room temperature followed by three washing steps, as recommended by the manufacturer. In the case of strawberries, two adaptations were made to the IMS protocol (preliminary study, results not shown). First, 100 µl wash buffer (phosphate buffered saline with tween 20; pH 7.4; Sigma Aldrich) was added to the 1 ml enriched BPW broth (pH 4.9 ± 0.6) to increase the capture efficiency of the beads. Second, the number of washing steps was reduced from three to two to obtain better recovery. Finally, 50 µl was plated on aCT-SMAC and aMAC for STEC O157 and O26, respectively, followed by incubation at 37°C for 18-24 h. In case of negative PCR signals, doubtful presumptive colonies on aCT-SMAC (with regard to morphology and colour), were plated on CHROMagar STEC. Whereas, in case of a positive PCR signal where no typical

colonies were achieved on aCT-SMAC or aMAC, the enrichment broth was plated on CHROMagar STEC without prior IMS.

2.3 Results

2.3.1 Blank samples

E. coli was not detected in any of the blank samples by Petrifilm (0/34). Also, no typical colonies of STEC O26 or *Salmonella* were isolated on aMAC (0/16) and SB (0/34) from non-inoculated samples (Table 5). In contrast, false positive results (presumptive colonies for STEC O157) were obtained with aCT-SMAC for all sample types (1/6 for strawberries, 3/6 for the lettuce mix and 5/6 for basil). In agreement with the negative PCR results, these suspected colonies were not confirmed to be STEC O157 on CHROMagar STEC, i.e. they did not display the typical colony characteristics. Also the growth of competing microbiota (noted as many non-typical blue and white colonies) could be easier distinguished from STEC strains on CHROMagar STEC, even in the case of strawberries, in contrast to aCT-SMAC and aMAC. In addition, 11/34 blank samples were PCR-positive for the *eae* gene with CT-values between 32.9 and 39.9, while negative for *stx1* or *stx2* (undetected or above the cut-off CT-value of 40.0), thus resulting in a negative result for STEC detection.

Two lettuce samples which gave a positive result for *Salmonella* with PCR (CT-values 33.3 and 35.6), while no typical colonies were detected by plating on SB. This discrepancy suggests that false positive results were obtained by *Salmonella* PCR. An alternative commercially available PCR kit, i.e. the Taqman *Salmonella enterica* detection kit (Life Technologies, Carlsblad, USA) showed negative PCR results, supporting the assumption of false positive (non-specific) PCR signals. Also the SB medium showed the growth and presence of competing microbiota as non-typical (blue) colonies for the lettuce (11/12) and basil (8/10) samples.

2.3.2 After one day of storage

Salmonella Thompson was detected on all fresh produce samples at all inoculum levels, with analysis by both the GeneDisc multiplex PCR and the culture method on SB (54/54 Table 5).

STEC O157 and O26 were detected by PCR in all fresh produce types and at the three inoculum levels used in this study (27/27 for both). However, no typical STEC O157 (grey-brownish with dark brown centre) or O26 (brown-reddish with red/violet centre) colonies were detected in four basil samples on aCT-SMAC or aMAC. Direct plating of those false negative samples yielded typical colonies on CHROMagar STEC. In addition, *E. coli* was only enumerated on Petrifilm from the samples inoculated with STEC O26.

Table 5: Detection of STEC O157 or O26 and *S.* Thompson inoculated on strawberries, a lettuce mix and basil stored for one or five days by PCR (GeneDisc Cycler) or culture media combined with or without IMS.

	Time (d)	Inoculation (cfu/25 g)	STEC O157				*S.* Thompson		STEC O26			*S.* Thompson	
			GeneDisc (PCR)			IMS +	GeneDisc	SB	GeneDisc (PCR)		IMS +	GeneDisc	SB
			stx2	*stx1*	*eae*	aCT-SMAC	(PCR)		*stx1*	*eae*	aMAC	(PCR)	
Strawberries	1	1000-7000	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		100-700	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		10-70	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		blank	0/3	0/3	2/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	5	1000-7000	2/3	2/3	2/3	2/3	2/3	2/3	3/3	3/3	3/3	3/3	3/3
		100-700	2/3	2/3	2/3	2/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3
		10-70	0/3	0/3	0/3	0/3	1/3	1/3	3/3	3/3	3/3	1/3	1/3
		blank	0/3	0/3	1/3	0/3	0/3	0/3	0/3	2/3	0/3	0/3	0/3
Lettuce mix	1	1000-7000	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		100-700	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		10-70	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		blank	0/3	0/3	1/3	0/3	0/3	0/3	0/3	1/3	0/3	1/3	0/3
	5	1000-7000	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		100-700	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		10-70	1/3	1/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		blank	0/3	0/3	0/3	3^P/3	0/3	0/3	0/3	1/3	0/3	1/3	0/3
Basil	1	1000-7000	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		100-700	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3
		10-70	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3
		blank[a]	0/3	0/3	1/3	2/3	0/3	0/3	0/2	0/2	0/2	0/2	0/2
	5	1000-7000	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3
		100-700	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		10-70	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3
		blank[a]	0/3	0/3	1/3	3^P/3	0/3	0/3	0/2	1/2	0/2	0/2	0/2

[a]: one missing result, [P]: presumptive colonies of STEC

2.3.3 After five days of storage

The recovery of the pathogens was lower on day five compared to day one, by both approaches of detection, i.e. as well by PCR as when attempting isolation by plating on appropriate selective agar media (Table 5).

In 8/54 inoculated samples *Salmonella* could no longer be detected by both approaches (PCR detection and plating on SB). All of the samples were strawberries and half of them were inoculated with the lowest level (10-70 cfu/25 g). Similar results were obtained for STEC: 7/54 inoculated samples no longer showed the presence of STEC. These were analyzed mostly from strawberries (5/7) in addition to some lettuce samples (2/7), the majority (5/7) being inoculated with the lowest level. Interestingly, all of these samples were inoculated with STEC O157, while STEC O26 was still detected under all tested conditions. In addition, two PCR negative lettuce samples for STEC showed typical colonies on aCT-SMAC. It should be noted that only *stx1* was detected instead of both *stx1* and *stx2* for one of the three lettuce samples inoculated with 100-700 cfu/25 g STEC O157 after five days of incubation, but the simultaneous detection of one *stx* gene and the *eae* gene was still regarded as a positive result. As for one day of storage, no typical STEC O157 or O26 colonies were detected in three basil samples on aCT-SMAC or aMAC after five days of storage, whereas these false negative samples yielded typical colonies on CHROMagar STEC. After five days, only samples inoculated with STEC O26 showed typical colonies for *E. coli* on the Petrifilm.

2.3.4 Inhibition during PCR

Inhibition of the PCR reaction was primarily observed for the basil samples, where 8 of the first 12 BPW enriched samples of basil analyzed were inhibited. After twofold dilution of the DNA extract of these samples, no inhibition was observed. Therefore, all other remaining basil samples (n = 34) were analyzed by using immediately a twofold dilution of the DNA extract. Three out of 34 basil samples still showed PCR inhibition. Finally, a tenfold dilution was able to overcome inhibition in all PCR reactions for basil samples. For strawberries and lettuce, 3/48 and 11/48 of the undiluted DNA extracts, respectively, showed PCR inhibition, which was overcome by a twofold dilution of DNA extract.

2.4 Discussion

Fresh fruits and vegetables (including fresh herbs) can become contaminated with *Salmonella* or STEC at all stages of production (Beuchat, 2002; Harris et al., 2003; Steele & Odumeru, 2004). However, unlike food from animal origin, there has been little attention to the performance of current detection methods for STEC and *Salmonella* in fresh produce (EFSA, 2013c; Jasson et al., 2009; Tzschoppe et al., 2012; Verstraete et al., 2012). The international standard detection method for *E. coli* O157 (ISO 16654:2001) is based on enriching the sample in modified TSB with novobiocin (mTSB + N), followed by IMS and plating on CT-SMAC and another selective media whereupon typical colonies must be confirmed. However, CT-SMAC does not differentiate sorbitol fermenting STEC O157 from other *E. coli* and was found to be less sensitive for samples of plant origin, as sorbitol fermenting Enterobacteriaceae may be present in high numbers on vegetables. For example, non-target strains like *Enterobacter cloacae*, *Salmonella* Enteridis, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* could grow on CT-SMAC, forming colourless colonies (Jinneman et al., 2012; Posse et al., 2008; Tzschoppe et al., 2012). Recently, ISO 13136:2012 has been published, describing a real-time PCR-based method for the detection of STEC (focusing on O157, O26, O103, O111 and O145) followed by an isolation and confirmation procedure, but no agreement exists on differential media for STEC, e.g. Tryptone Bile X-glucuronide (TBX) recommended in ISO 13136:2012 is a selective, chromogenic medium for all *E. coli* strains. In contrast to classical STEC O157, non-O157 strains have no unique biochemical characteristics to distinguish them from other *E. coli* strains. Posse et al. (2008) developed differential media for non-O157 STEC (O26, O103, O111 and O145) based on utilization of different carbohydrates. All STEC strains of the serotypes O26, O103, O111 and O145 that were tested could be isolated from mixtures with commensal *E. coli* strains and none of the non-target strains were able to grow on the differential media or they showed a different colony morphology than the target STEC strains. However, with the adaptation of CT-SMAC and MAC, as recommended by (Posse et al., 2008), it was still a challenge to identify presumptive positive colonies, mainly for the basil samples. Also in Verstraete et al. (2012) the recovery rate of STEC on sprouted seeds was extremely low (1/60 to 5/30 depending on the inoculum) due to overgrowth of background microbiota on selective agars. Hence, enriched samples were plated on

CHROMagar STEC by means of a sterile loop without prior IMS, after which typical STEC colonies were obtained, indicating that this medium might be more suitable for the detection of the used STEC strains in basil. Tzschoppe et al. (2012) reported that samples plated onto CHROMagar STEC (without applying IMS) showed exhaustive growth of Enterobacteriaceae and *Pseudomonas* for enriched spiked salad samples in BPW, which made it difficult to isolate presumptive STEC colonies. However, CHROMagar STEC was found to be more sensitive than CT-SMAC in case of vegetable samples, as confirmed by our study. But, it should be noted that several STEC strains, especially sorbitol fermenting STEC O157 and STEC O103 and some O26:H11 STEC strains, could not grow on CHROMagar STEC (Hirvonen et al., 2012; Tzschoppe et al., 2012). In addition, Weagant et al. (2011) showed the importance of using several isolation media as false-negative results were obtained on all investigated media (Rainbow Agar O157, R and F *E. coli* O157:H7 agar and CT-SMAC) in case of alfalfa sprouts.

IMS is a useful procedure for extracting target pathogenic bacteria from food sample enrichments, using magnetic beads coated with antibodies to capture the target bacteria. Detection of low inoculum levels of *E. coli* O157:H7 on alfalfa sprouts by plating was improved after IMS in comparison with direct plating (Weagant et al., 2011). In addition, *E. coli* O157:H7 was better recovered from alfalfa sprouts by PCR when using IMS in advance than without IMS (Fedio et al., 2012). However, in the present study, 7 PCR positive results for STEC in basil were negative after IMS and plating on aCT-SMAC (3/7) or aMAC (4/7), whereas positive results (7/7) were obtained after direct plating of these samples on CHROMagar STEC. In agreement with our results, STEC was isolated using the direct plating method but not when IMS was applied (Verstraete et al., 2012). Therefore, contradictory results regarding the use of IMS are currently reported, thus further research on this topic is warranted.

In case of *Salmonella* the standard ISO 6579:2002 detection method may not perform optimally if *Salmonella* is present in low numbers amongst competing microbiota or if subjected to adverse growth environment. However, clear results were obtained for *Salmonella* on SB medium in this study. Presumptive *Salmonella* colonies were easy to distinguish from background flora, which was mainly present in the samples of lettuce and basil.

Molecular detection methods, e.g. PCR, are increasingly applied as alternative detection methods in food microbiology. A need exists for more reliable and faster detection than the currently available culture methods, especially for STEC detection in fresh produce. However, attention should still be paid to the potential occurrence of false positive and false negative results when using PCR. False positive results can be obtained by cryptic target genes (such as free *stx* phages) or by nonspecific amplification (Bettelheim, 2003). In this study, two false positive results for *Salmonella* were obtained with GeneDisc PCR. Using STEC PCR, the *eae* gene was found in 11/34 blank samples. The *eae* gene, coding for intimin, is present in pathogenic *E. coli*, but it was reported to be present in other bacteria as well, such as *Citrobacter rodentium* (Beutin et al., 2009). The results of our study confirm the occurrence of *eae* in bacteria constituting the natural microbiota of fruits and vegetables, but not in combination with the toxin genes *stx1* and/or *stx2*, so no false positive results were obtained by the conducted STEC PCR. Moreover, it has been shown that *stx* variants have been expressed by *S. dysenteriae, Enterobacter, Citrobacter, Acinetobacter* and *Aeromonas* (Mauro & Koudelka, 2011). False negative results can be caused by PCR inhibition or DNA degradation through plant polysaccharides, phenolic compounds, DNases, etc., present in the enrichment broth (Wilson, 1997). Inhibition was observed more frequently for basil samples, probably due to the presence of phenolic compounds (Jayasinghe et al., 2003; Leal et al., 2008), while in lettuce and strawberries, phenolic compounds are present in lower amounts (Aaby et al., 2012; Khanam et al., 2012; Tiveron et al., 2012). By 1:2 dilution (or in some cases of basil 1:10), PCR inhibition was resolved without an impact on the performance of the method and the ability to detect the inoculated pathogens in the present study. Moreover, prior stress and injury or the presence of competing microbiota could impair or suppress the growth of the target bacteria during enrichment and thus lead to false negative PCR results (Verstraete et al., 2012). A third cause of false negative results is the spontaneous loss of *stx* genes after the first or multiple subcultivation steps or long preservation (Brooks et al., 1997; Feng et al., 2001; Joris et al., 2011b; Karch et al., 1992), because these genes are encoded on temperate bacteriophages (Schmidt, 2001). In the present study, the loss of *stx2*, whereas *stx1* and *eae* were still detected, was observed in one lettuce sample inoculated with STEC O157.

Overall results of STEC and *Salmonella* detection obtained using the culture methods were comparable with those of the GeneDisc multiplex PCR. Negative samples by GeneDisc detection were confirmed to be negative by using the culture methods, except for the two blank lettuce samples which showed a PCR positive result for *Salmonella* that could not be confirmed by isolation. In addition, one blank strawberry sample, three blank lettuce samples and five blank basil samples showed STEC presumptive colonies on aCT-SMAC agar, but not on CHROMagar STEC. Fresh produce is a challenging food matrix for the detection of low numbers of pathogens because of the presence of a wide range of indigenous competing microbiota and adverse conditions for survival of these pathogens. Therefore, it is necessary to use multiple types of detection methods and multiple selective agar media for estimating the presence of pathogens in these types of fresh produce, as indicated in our results. Positive samples were usually detected as such by both PCR and culture methods, except for some observed false negative results with PCR on two lettuce samples inoculated with 10-70 cfu/25 g of STEC O157 after five days of storage and some observed failures in isolation of the pathogenic strains by the culture method for 7 basil samples inoculated with various levels of STEC O157 and O26 after one and five days of storage by plating on either aCT-SMAC or aMAC. Most of the problems for PCR detection (inhibition) and culture isolation (growth of background microbiota on agar media) were noted for basil samples.

The *S.* Thompson, STEC O157 and O26 strains in the present study were detected at all three inoculum levels after one day of storage on all included fresh produce samples and also after five days for the lettuce and basil samples, but they were no longer consistently detected on the strawberry samples after five days. The recovery rate of *S.* Thompson after five days of storage on strawberries was 56% (10/18) and that of STEC (combined for O157 and O26 STEC) was 72% (13/18). Interestingly, reduced recovery of STEC was only observed for STEC O157 (44%, 4/9), while STEC O26 was still detected (100%, 9/9). The latter may suggest superior survival potential for the STEC O26 strain after prolonged storage. These results suggest that considerable strain variation exists in the survival capacity of pathogenic *E. coli* on strawberries during cold storage.

This study indicates the ability of a PCR based screening method for reproducible multi-detection of low numbers (10-70 cfu/25 g) of STEC and *Salmonella* in mixed lettuce, basil and strawberries although in the basil samples, PCR needed twofold dilution of the DNA extract

to overcome inhibition. It remains a challenge to consistently detect and isolate STEC and *Salmonella* strains after PCR screening, especially to detect low numbers in these type of fresh produce with either high numbers of indigenous microbiota or having adverse (e.g. acid) conditions for growth and survival of these enteric pathogens. IMS did not necessarily contribute to improve recovery of the STEC, whereas the use of an additional agar medium improved recovery rate of STEC in the present study. The appropriateness of this detection approach will need further verification in surveys with naturally contaminated samples.

CHAPTER 3: Survival of *Salmonella* and *E. coli* O157:H7 on strawberries, basil and other leafy greens during storage

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ABSTRACT

The survival of *Salmonella* and *Escherichia coli* O157:H7 on strawberries, basil leaves and other leafy greens (spinach, lamb and butterhead lettuce leaves, baby leaves and fresh-cut iceberg lettuce) was assessed at cold (< 7°C) and ambient temperatures. All commodities were spot inoculated with *Salmonella* or *E. coli* O157:H7 to obtain an initial inoculum of 5 to 6 log cfu/g and 4 to 5 log cfu/g for strawberries and all types of leafy greens respectively. Samples were air-packed. Strawberries were stored at 4°C, 10°C, 15°C, and 22°C and basil leaves and other leafy greens at 7°C, 15°C and 22°C for up to 7 days (or less if spoiled before). Both *Salmonella* and *E. coli* O157:H7 showed a gradual decrease in numbers if inoculated on strawberries, with a similar reduction observed at 4°C, 10°C and 15°C (2 to 3 log units after 5 days). However, at 15°C (and 10°C for *E. coli* O157:H7) the survival experiment stopped before day 7 as die-off of both pathogens below the lower limit of detection was achieved or spoilage occurred. At 22°C strawberries were moldy after 2 or 4 days. At that time a 1 to 2 log reduction of both pathogens had occurred. A restricted die-off (on average 1.0 log unit) and increase (on average < 0.5 log unit) of both pathogens on basil leaves occurred after 7 days storage at 7°C and 22°C respectively. On leafy greens a comparable decrease as on basil was observed after 3 days at 7°C. At 22°C both pathogens increased to higher numbers on fresh-cut iceberg and butterhead lettuce leaves (on average 1.0 log unit), probably due to the presence of exudates. But, by using spot inoculation the increase was rather limited probably due to minimized contact between the inoculum and cell exudates. Avoiding contamination in particular at cultivation (and (post-)harvest) is important as both pathogens survive during storage and strawberries, basil and other leafy green leaves are consumed without inactivation treatment.

3.1 Introduction

During postharvest storage, refrigeration and high relative humidity conditions (90-95%) are recommended for berries (e.g. strawberries) and leafy greens (e.g. lettuce and fresh herbs) to reduce respiration, water losses, ethylene production and microbial development. For basil, refrigeration at temperatures lower than 10°C is unsuitable due to development of chilling injuries (blackening) (Cantwell, 2001; Lange & Cameron, 1994). Foodborne outbreaks occurred with strawberries and basil after transport and storage, suggesting survival of *Salmonella* or *E. coli* O157:H7 on both commodities, even in adverse environments (Laidler et al., 2013; Pakalniskiene et al., 2009; Pezzoli et al., 2008).

Although survival of *Salmonella* and *E. coli* O157:H7 have been studied on leafy greens such as spinach (Luo et al., 2009; Sant'Ana et al., 2012), escarole (Abadias et al., 2012; Sant'Ana et al., 2012) and, especially, on fresh cut iceberg or romaine lettuce (Chang & Fang, 2007; Francis & O'Beirne, 2001; Gleeson & O'Beirne, 2005; Weissinger et al., 2000; Zeng et al., 2014) and growth models are available for iceberg lettuce (Koseki & Isobe, 2005; McKellar & Delaquis, 2011) to predict growth of *E. coli* under temperature abuse, limited information is available about the survival of *Salmonella* and *E. coli* O157:H7 on strawberries and basil leaves during storage. No information about the survival of *Salmonella* or *E. coli* O157:H7 on basil leaves during storage at 15°C is available, which is the recommended temperature to maintain quality during storage. In case of strawberries, the time/temperature conditions which have been studied during storage experiments so far are rather limited (Knudsen et al., 2001; Yu et al., 2001).

The present study aimed to study the survival of *Salmonella* and *E. coli* O157:H7 on fresh strawberries and basil leaves during storage at temperatures corresponding to retail and household refrigerators (4°C and 7°C), basements (10°C and 15°C) and domestic kitchens (22°C) for up to one week or shorter if spoiled before in their commercial packages. In addition, the behavior of these pathogens was assessed on spinach, lamb and butterhead lettuce leaves, baby leaves and fresh-cut iceberg lettuce during three days storage at 7°C, 15°C and 22°C to compare with the survival on basil leaves.

3.2 Material and methods

3.2.1 Bacterial strains

Two *E. coli* O157:H7 strains and two *Salmonella* strains were used to assess the survival on strawberries and leafy greens. *E. coli* O157:H7 strain LFMFP 846 was isolated from beef carpaccio and obtained from the Institute for Agricultural and Fisheries Research (ILVO, Belgium). The strain was *stx* negative. *E. coli* O157:H7 strain CECT 5947 was an attenuated (*stx* negative) strain obtained from the Spanish Type Culture Collection (CECT). The nalidixic acid resistant version of both strains obtained as described in (Lopez-Galvez et al., 2012), was used in the experiments. *Salmonella enterica* serovar Thompson RM1987N is a spontaneous nalidixic acid resistant mutant of *Salmonella* Thompson strain RM1987 and was received from Dr. Maria Brandl (ARS USDA, Albany, California, USA). Strain RM1987 is a previously described clinical isolate from a patient in a cilantro-linked outbreak in California (Brandl & Mandrell, 2002; Brandl et al., 2005). *Salmonella enterica* serovar Typhimurium strain (SL 1344) was a reference strain obtained from Dr. Maria Brandl (Brandl et al., 2011).

The strains, stored at -75°C on glass beads, were revived in 10 ml Brain Heart Infusion broth (BHI, Oxoid, UK) for 24 h at 37°C and grown on BHI slants (37 g/l BHI and 16 g/l bacteriological agar (Oxoid)) for 24 h at 37°C. BHI slants were kept in the fridge for 1 month. Only in the case of *E. coli* O157:H7, nalidixic acid (50 µg/ml) was added to the BHI (broth and slant) to retain their resistance, which was needed for the isolation/enumeration (to suppress growth of background microbiota). In case of *Salmonella* this was not needed because of the availability of more selective culture media such as Xylose-Lysine-Desoxycholate agar(XLD) and *Salmonella* Brilliance.

3.2.2 Inoculation procedure

Strains were revived from BHI slants by culture in 10 ml BHI broth for 24 h at 37°C followed by sub-culturing 100 µl of the 24 h culture in another 10 ml BHI (24 h; 37°C). Nalidixic acid (50 µg/ml) was added to the BHI in case of *E. coli* O157:H7. Before inoculation, both strains of *E. coli* O157:H7 and both strains of *Salmonella* were mixed. Subsequently, both mixtures were washed to avoid the influence of the nutrients present in BHI on the survival (observed during a trial study). Therefore, 1 ml of BHI broth mixture was centrifuged (1 min, 14,000

rpm) using a micro tube centrifuge (5414 C; Hinz GmbH, Germany) followed by the discard of the supernatants and the suspension of the pellet in 1 ml distilled water by using a vortex. Previous steps were repeated until the pellet was washed three times and finally the pellet was suspended in 1 ml distilled water. Each sample was inoculated with 100 µl of the washed *E. coli* O157:H7 or *Salmonella* inoculum by means of spot inoculation (droplets of 5 µl). The survival of both pathogens on strawberries and basil was followed for up to 7 days (or less if visual spoilage occurred before, e.g. appearance of molds on strawberries or presence of limp or wilted leaves on leafy greens) or the pathogens on all three samples dropped below the detection limit, i.e. 50 cfu/g for strawberries and 100 cfu/g for leafy greens), whereas on the other leafy greens for 3 days. Each temperature/time condition was provided in triplicate and in case of strawberries and basil two independent storage experiments were performed (study A and B). Study A was performed during March/April, whereas study B was performed during November/December.

Strawberries (*Fragaria*) were obtained from retail in Belgium and packed in perforated boxes (100 ± 5 g). Only strawberries without visual quality defects were used. The inoculum (100 µl) was distributed on the fruit, not on the calyx, and after inoculation the samples were dried for 30 min in the biosafety cabinet at ambient temperature (approx. 20°C). Subsequently, samples were incubated at different temperatures, mimicking correct refrigeration (4°C), refrigeration but with temperature abuse (10°C), storage in basement (15°C) and storage at room temperature (22°C).

Basil (*Ocimum basilicum*) leaves (in closed trays of 25 g) were obtained from retail and only packages free from visual defects was used for storage experiments. The inoculum was put between the leaves using a forceps to avoid contact with the packaging. In contrast to strawberries inoculated basil samples were not dried, as quality defects (dehydration) were observed during a preliminary study. Samples were stored at 7°C (refrigeration with temperature abuse (chilling injuries when basil stored below 10°C)), 15°C (storage in basement) and at 22°C (room temperature).

Finally, the survival of *Salmonella* and *E. coli* O157:H7 was assessed on fresh-cut iceberg lettuce (*Lactuca sativa* var. *capitata* L. *nidus jäggeri* Helm), butterhead lettuce leaves (*Lactuca sativa* var. *capitata* L. *nidus tenerrima* Helm*)*, baby leaves, baby spinach leaves

(*Spinacia oleracea*) and lamb lettuce leaves (*Valerianella locusta*) as a comparison to basil leaves. Baby leaves are a mixture of young lettuce leaves such as lollo bioda, endive, red roman, etc. All leafy greens were obtained from retail. Iceberg lettuce was cut in squares (3 cm x 3 cm) and leaves of a butterhead lettuce crop were cut at the base of the crop but left as whole leaves. The rest of the leafy greens remained as small intact leaves as bought in retail. Twenty-five gram of each commodity was put in suitable packages to keep air conditions and avoid significant decreases of O_2 concentration during storage due to respiration. For fresh-cut iceberg lettuce and lamb lettuce the packaging size was 15 x 15 cm², whereas for the other leaves 18 x 18 cm² was used. The permeability of the plastic film for the package was 2200 ml O_2/m²/d/atm (7°C; Amcor Flexibles, UK) for iceberg lettuce, 4600 ml O_2/m²/d/atm (7°C; Amcor Flexibles, UK) for butterhead lettuce and lamb lettuce leaves and 2.0 ml O_2/m²/d/atm (23°C; Euralpack, Belgium) for baby leaves and baby spinach leaves. All packages were closed by heat sealing except for baby leaves and baby spinach where a vent was left to permit exchange of O_2 and CO_2. The survival of both pathogens on the different leafy greens was monitored during storage at 7°C, 15°C and 22°C. In the latter case, the survival was assessed during 3 days of storage and only one experiment was performed.

3.2.3 Microbiological analysis and visual quality assessment

The whole sample (100 g and 25 g for strawberries and leafy greens, respectively) was respectively 5- and 10-fold diluted in Peptone Physiological Salt Solution (PPS; 1 g/l neutralized bacteriological peptone (Oxoid, UK) and 8.5 g/l NaCl (Sigma Aldrich, Germany)) in a stomacher filter bag and stomached for 1 min. After making a 10-fold serial dilution in PPS, *E. coli* O157:H7 and *Salmonella* were enumerated by plating on Chromocult agar (Merck KGaA, Germany) with addition of nalidixic acid (NA, 50 µg/ml) and on Xylose-Lysine-Desoxycholate agar (XLD; Oxoid), respectively. Both agar plates were incubated at 37°C for 24 h ± 3 h. Suspected *E. coli* O157:H7 and *Salmonella* colonies were indicated respectively as pink colonies on Chromocult + NA and as red transparent colonies with a black center on XLD.

In addition, the visual quality of the strawberries and basil and other leaves was assessed by the technician on the samples about to be processed for microbiological analysis by a 5-

point or 9-point hedonic scales, with the acceptance limit at 3 and 5 respectively (Kader et al., 1973). A score of 1 (5-point) and 9 (9- point) means respectively “not affected” and “like it very much”, while a score of 5 and 1 means “severely affected” and “dislike it very much”. Different parameters were taken into account: (i) off-odor, freshness and general visual quality for strawberries, (ii) chilling injuries, appearance and general visual quality for basil and (iii) browning (due to oxidation of phenolic substances) or decay (e.g. bacterial soft rot), appearance (physiological deterioration as wilting and water loss; not applicable for iceberg lettuce) and overall visual quality for the other leafy greens. Overall visual quality was scored by the 9-point scale, the other parameters by the 5-point scale. Samples, that were not acceptable anymore for consumption (beneath acceptance limit) due to growth of molds or decay, were discarded and not analyzed for the microbiological parameters described above. When only a small mold on one of the strawberries in the package was observed, samples were still analyzed (but the molded strawberry was removed) as the worst case was taken into account, i.e. the consumer removes the strawberry which has a small mold and consumes the other strawberries of the package which were still acceptable.

3.2.4 Statistical analysis

Log reductions (the average log cfu/g on day 0 minus the log cfu/g on day x) for both pathogens on each produce-temperature combination were calculated and used for statistical testing. Day x indicates the last day where all replicate (3) samples could be analyzed (e.g. no replicates removed from the experiment due to spoilage). Statistical analysis was performed in SPSS (version 22) and due to the small sample sizes non-parametric tests (Kruskal-Wallis) were used with a 5% significance level (and thus a confidence level of 95%). If a significant effect was obtained with Kruskal-Wallis, pairwise comparisons were performed and the Bonferroni correction was applied to obtain a family-wise confidence level of at least 95%.

3.3 Results

3.3.1 Visual quality of strawberries, basil leaves and other leafy greens during storage

During storage the visual quality was monitored and only samples that were acceptable for consumption were analyzed. For strawberries stored at 22°C, the end of the experiment was reached after 2 (study A) or 4 (study B) days of storage due to the growth of molds and rot (brown spots) on all samples. In addition the acceptance limit of freshness (score = 3) was reached after 2 (A) or 3 (B) days in 6 and 5 of the 6 samples respectively, while the odor only changed substantially at day 4 in study B (score = 3). Storage at 15°C resulted in growth of molds from day 5 (A) or 4 (B) and rot from day 6, limiting the experiment until 6 days in both studies. The acceptance limit of freshness was reached after 5 days in more of half of the analyzed samples, of which 2 samples showed a change in odor (score = 3). When stored at 4°C and 10°C strawberries remained acceptable for one week, only a decrease in freshness was noticed. A single mold in some of the packages was observed from day 3 on at 4°C and 10°C.

In contrast to strawberries, the survival on basil leaves could be monitored for the full week at all temperatures. Only when stored at 22°C, not all samples remained acceptable for consumption due to growth of molds, especially in study B where the end of shelf life was reached after 6 days (the first mold was already observed after 3 days). Also at 15°C molds started to develop after 5 days. Chilling injuries was mainly noticed at 7°C from day 4 on in study A, but was rather limited. A decrease in appearance (i.e. more wilting or water loss) was noticed from day 2 on at all temperatures (also mainly in study A).

The overall visual quality during storage decreased the most for lamb lettuce, especially at 15°C and 22°C. After 3 days storage at 22°C lamb lettuce reach the acceptance limit for consumption, due to decay. The other leafy greens remained acceptable after 3 days storage, however some browning was noticed on iceberg lettuce, especially at 15°C and 22°C. Also the appearance decreased the most when leafy greens were stored at 22°C and especially for lamb lettuce. The least decrease in appearance was observed for baby leaves.

3.3.2 Survival of *Salmonella* and *E. coli* O157:H7 on strawberries.

Both pathogens died off during storage on strawberries at all temperatures; after 7 days at 4°C the initial numbers were decreased with 2.5 to 3.9 log units (Table 6, Figure 9). When storage at 10°C, *Salmonella* was still present after one week, although a decrease of 2.5 to 3.9 log units was observed. On the other hand in both studies no *E. coli* O157:H7 could be enumerated anymore after 6 days storage at 10°C (decrease of 3.4 to 3.8 log units).

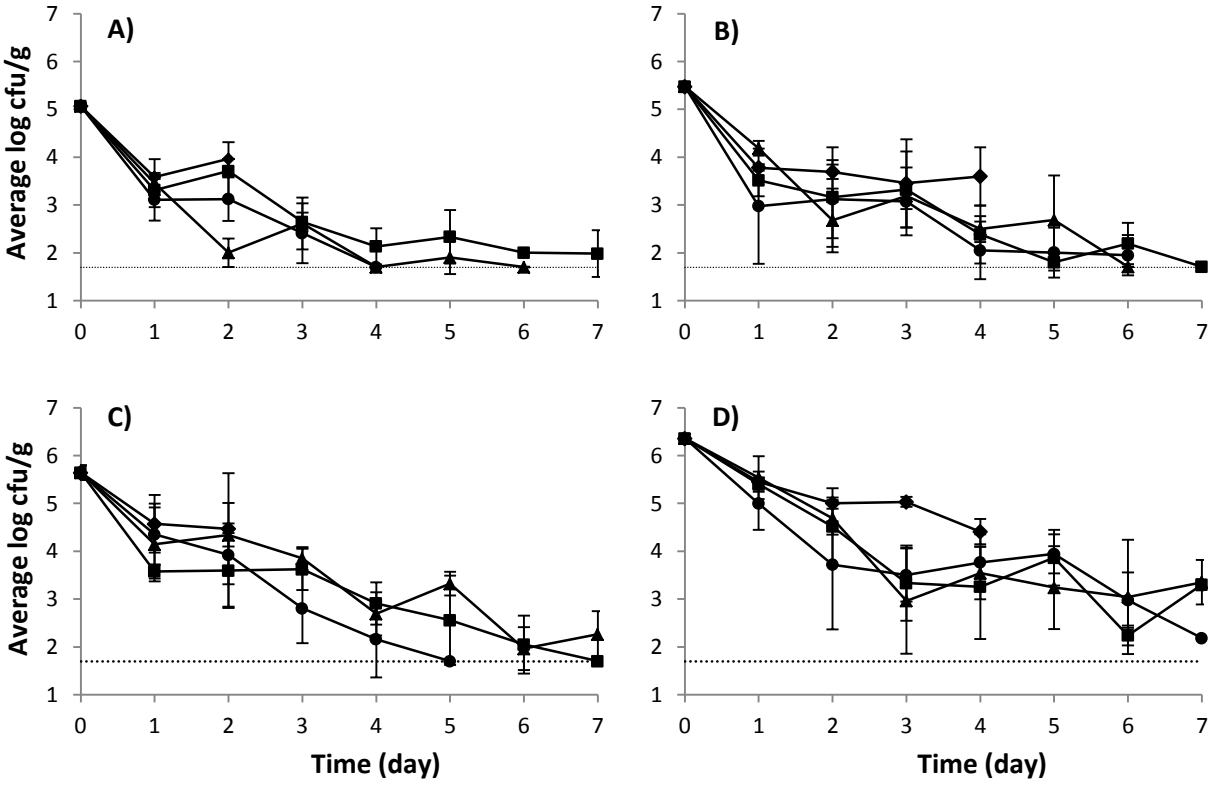

Figure 9: Survival of *E. coli* O157:H7 (A & B) and *Salmonella* (C & D) on strawberries stored at 4°C (■), 10°C (▲), 15°C (●) and 22°C (◆) for 7 days. Two experiments were performed (study A (A & C) and study B (B & D)) and each data point is the average of 3 samples (n = 3). Error bars display the standard deviation.

At 15°C, the number of pathogens dropped below the limit of detection of the enumeration method (1.7 log cfu/g) after 4 and 5 days for *E. coli* O157:H7 and *Salmonella*, respectively, in study A. In study B at 15 °C, *E. coli* O157:H7 and *Salmonella* could still be enumerated until day 6, when the end of shelf life was reached due to the growth of molds and rot. At 22°C the experiment was limited until 2 (study A) or 4 (study B) days due to growth of molds and rot. A die-off of on average 1.1 (study A) to 1.8 (study B) and 1.2 (study A) to 1.4 (study B) log units was observed after 2 days storage at 22°C in case of *E. coli* O157:H7 and *Salmonella,*

respectively. However, no significant difference in survival was found between the two experiments A and B (*E. coli* O157:H7 p = 0.389; *Salmonella* p = 0.272). Only in terms of quality (molds or rot) a difference was noticed, strawberries from the second experiment remained fresh for a longer time and molds developed later. At all temperatures a decrease was noticed during storage, but for *E. coli* O157:H7 significantly less die-off was observed at 22°C compared to 15°C (p = 0.035), 10°C (p = 0.015) and 4°C (p = 0.006) and for *Salmonella* at 22°C compared to 15°C (p = 0.006) and 4°C (p = 0.009).

Table 6: Number of strawberry samples with enumerable (≥ 50 cfu/g) *E. coli* O157:H7 and *Salmonella* of the total number inoculated (n = 3) during one week storage in study A and B. When no pathogens were enumerated anymore, the detection limit (1.7 log cfu/g) was used to calculate the average and standard deviation in Figure 9 (ND = not done)

	Day	1		2		3		4		5		6		7	
	Study	A	B	A	B	A	B	A	B	A	B	A	B	A	B
***E. coli* O157:H7**	**4°C**	3	3	3	3	3	3	2	3	3	1	3	2	1	1
	10°C	3	3	2	3	3	3	1[a]	3	1	2	0	0	ND	ND
	15°C	3	3	3	3	3	3	0	3	ND	2	ND	1	ND	0[b]
	22°C	3	3	1[b]	3	ND	3	ND	3	ND	ND	ND	ND	ND	ND
Salmonella	**4°C**	3	3	3	3	3	3	3	3	2	3	1	2	2	3
	10°C	3	3	3	3	3	3	3	3	3	3	2	2	2	3
	15°C	3	3	3	3	3	3	1	3	0[a]	3	ND	3	ND	1[b]
	22°C	3	3	3	3	ND	3	ND	3	ND	ND	ND	ND	ND	ND

[a]1 missing result, [b] 2 missing results due to the growth of molds and rot

3.3.3 Survival of *Salmonella* and *E. coli* O157:H7 on basil

Salmonella and *E. coli* O157:H7 survived during one week storage at all temperatures (7°C, 15°C and 22°C) (Figure 10). Storage at 7°C resulted in a decrease of 0.4 to 1.6 and 0.3 to 1.8 log units for *E. coli* O157:H7 and *Salmonella* after one week respectively. At 15°C a slight decrease (< 0.6 log units) or increase (< 0.9 log units) was observed for *E. coli* O157:H7, while for *Salmonella* a similar decrease as at 7°C was noticed (0.5 to 1.7 log units). When stored at 22°C, 4 samples were discarded at day 7 due to the growth of molds and rot in study B, limiting the experiment to 6 days in case of *Salmonella*. After 6 or 7 days storage at 22°C, the increase/decrease of *E. coli* O157:H7 and *Salmonella* was ≤ 1 log unit. As described before, no significant difference was observed between experiment A and B (*E. coli* O157:H7 p =

0.389; *Salmonella* p = 0.272). For *E. coli* O157:H7 a significant difference was found between 22°C and 7°C (p = 0.001), whereas for *Salmonella* a significant difference between the temperatures (p = 0.045) was found which could not be attributed to specific temperature pairs by further pair-wise comparison, probably due to the low sample size combined with the conservative Bonferroni correction.

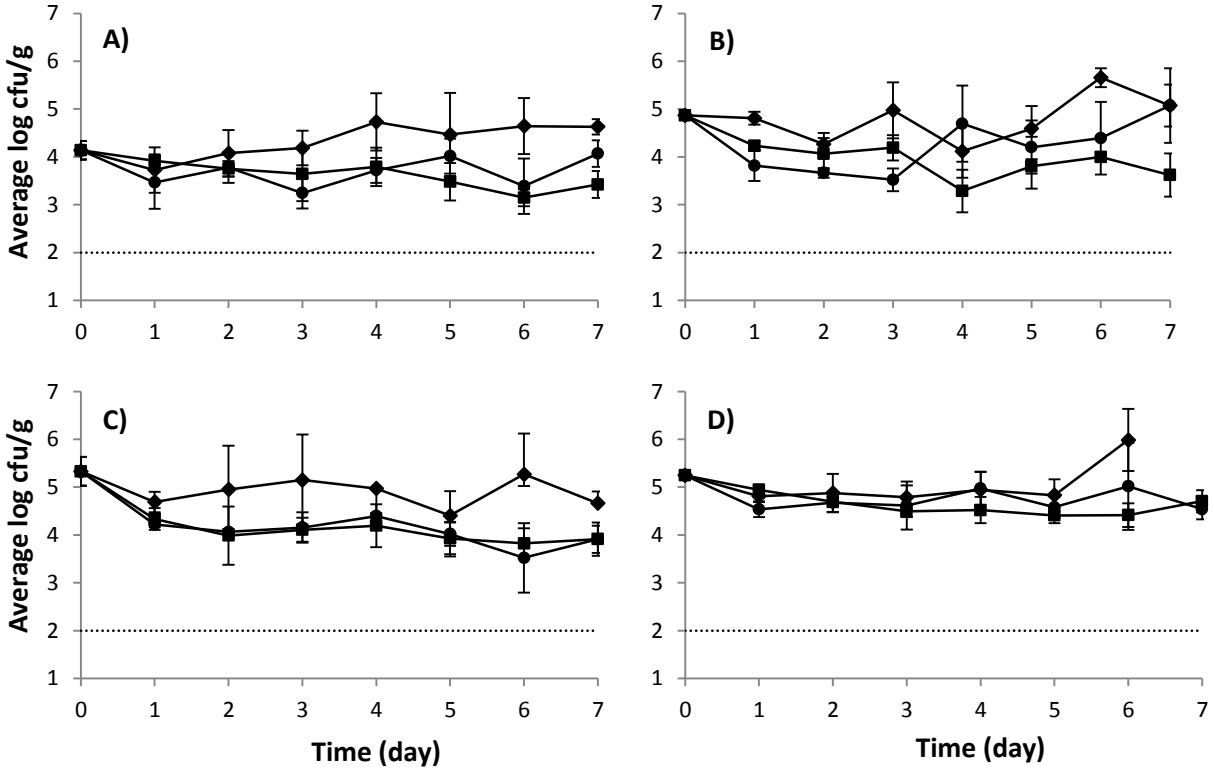

Figure 10: Survival of *E. coli* O157:H7 (A & B) and *Salmonella* (C & D) on basil stored at 7°C (■), 15°C (●) and 22°C (◆) for 7 days. Two experiments were performed (study A (A & C) and study B (B & D)) and each data point is the average of 3 samples (n = 3). Error bars display the standard deviation.

3.3.4 Survival of *Salmonella* and *E. coli* O157:H7 on some leafy greens

To verify whether the lack of substantial outgrowth of both pathogens on basil stored at 22°C after 7 days of storage is a specific characteristic of basil, the survival of both pathogens was compared to the survival on different leaves of other leafy greens, including lamb lettuce, spinach, baby leaves, butterhead lettuce and, as a reference for acknowledged growth of pathogens, behavior of pathogens on fresh-cut iceberg lettuce was monitored. No marked difference in survival of both pathogens on the different commodities was noticed after 3 days storage. Only on lamb lettuce a significantly larger decrease of *E. coli* O157:H7

was observed compared to fresh-cut iceberg lettuce at 15°C (p = 0.007) and at 22°C (p = 0.012) and to butterhead lettuce at 22°C (p = 0.033). Also for *Salmonella* a significantly larger decrease on lamb lettuce was observed at 15°C compared to butterhead lettuce (p = 0.010) and at 22°C compared to iceberg lettuce (p = 0.017) (Figure 11).

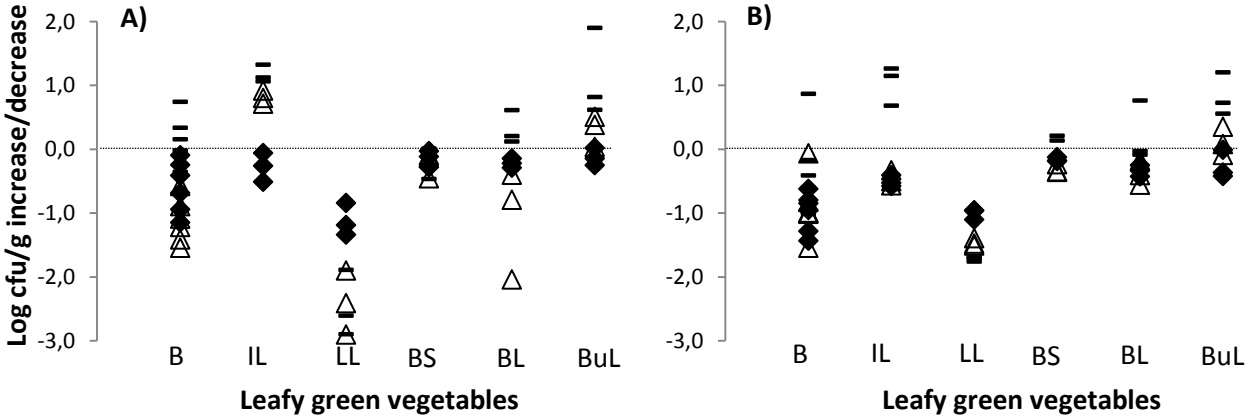

Figure 11: Log change (count on day x (log cfu/g) – average of counts on day 0 (log cfu/g)) of *E. coli* O157:H7 (A) and *Salmonella* (B) on basil (B), fresh-cut iceberg lettuce (IL), lamb lettuce (LL), baby spinach (BS), baby leaves (BL) and butterhead lettuce (BuL) after 3 days storage at 7°C (◆), 15°C (△) and 22°C (-). Positive and negative values indicate increase and decrease respectively.

At 7°C no substantial difference in the behavior of *E. coli* O157:H7 and *Salmonella* between all leafy greens was observed, only in case of *Salmonella* a significantly higher decrease was observed on basil compared to baby spinach (p = 0.041). A significant difference between 22°C and 7°C was found on fresh-cut iceberg lettuce (p = 0.021) and butterhead lettuce (p = 0.026) for *E. coli* O157:H7 and on lamb lettuce (p = 0.020) and butterhead lettuce (p = 0.033) for *Salmonella.* In addition, a significant difference was found between 22°C and 15°C on baby spinach (p = 0.036) for *Salmonella* and on baby leaves (p = 0.022) and on basil (p = 0.002) for *E. coli* O157:H7 after 3 days storage.

3.4 Discussion

In the present study spot inoculation was preferred above dip inoculation as this method is more consistent (Lang et al., 2004). In addition, spot inoculation mimics more accurately point contamination (e.g. contact with animal feces, hands or soil) and less free water is present, which promotes survival or growth of inoculated bacteria and the germination of molds spores (Brandl, 2006; Knudsen et al., 2001). Several factors have an influence on microbial growth during storage, e. g. temperature, relative humidity and nutrient availability (Devlieghere et al., 2013). The storage temperature is known to be an important factor affecting the survival or growth of pathogens and the quality of fresh produce. At abusive temperature (> 7°C) growth of *Salmonella* and *E. coli* O157:H7, on fresh produce is possible. But higher temperatures can facilitate rapid quality decline due to the proliferation of spoilage bacteria or an increase in physiological deterioration shortening the shelf life (Jacxsens et al., 2002).

Strawberries are highly perishable fruits, susceptible to mechanical injuries and microbiological decay (especially growth of molds such as *Botrytis cinerea*) (Hernandez-Munoz et al., 2006). Therefore, strawberries are harvested manually and not washed after harvesting, as water promotes the growth of molds. Moreover, strawberries are consumed raw, which increases the potential risk of foodborne illness for consumers. In 2011, an *E. coli* O157:H7 outbreak occurred in Oregon with strawberries which were contaminated by feces of a deer (Laidler et al., 2013). The fruit does not support the growth of pathogens due to the low pH (a pH between 3.3 and 3.5 was measured in the present study) and the dry and somewhat waxy surface (Knudsen et al., 2001). However, some pathogens are tolerant to acidic conditions as observed for *E. coli* O157:H7 in strawberry juice (Han & Linton, 2004) and apple cider (Roering et al., 1999) and *Salmonella* in cut tomatoes (Asplund & E., 1991). But, through storage of strawberry juice at cold temperature (4°C) *E. coli* O157:H7 cells became injured, whereas a higher storage temperature (37°C) resulted in inactivation of *E. coli* O157:H7 after 3 days (Han & Linton, 2004). Moreover, survival depends also on the type of acidulants (Conner & Kotrola, 1995). In the present study, no growth, only inactivation, was observed for *E. coli* O157:H7 and *Salmonella* during storage on fresh whole strawberries at all temperatures (ranging from 4°C to 22°C). For the storage of strawberries, refrigerated

temperatures are recommended (Cantwell, 2001). Nevertheless, both *E. coli* O157:H7 and *Salmonella* were still present above the detection limit for enumeration (1.7 log CFU/g) after inoculation with 5 to 6 log CFU/g and storage at 4°C for one week in respectively 2 out of 6 and 5 out of 6 samples. Also in Knudsen et al. (2001), *E. coli* O157:H7 and *Salmonella* survived during one week storage at 5°C but with less decrease (1 to 2 log units) than observed in the present study. In Yu et al. (2001) *E. coli* O157:H7 decreased with 1.3 to 1.8 log units at 5°C and 2.1 to 2.3 log units at 10°C after 3 days storage, which is slightly less compared to our present study. At room temperature, no substantial increase or decrease of *E. coli* O157:H7 and/or *Salmonella* was observed in the study of Knudsen et al. (2001) and Yu et al. (2001) after 48 h and 24 h respectively, unlike the present study. In Yu et al. (2001) and Knudsen et al. (2001) molds started to develop after 5 days at 7°C and 7 days at 5°C respectively, while in our study the first molds were visible after 3 days of storage at 4°C or 10°C. At 24°C, the experiment had to be discontinued in Knudsen et al. (2001) after 2 days due to the growth of molds, as found in the present study.

Basil is commonly used fresh (e.g. as decoration in a ready-to-eat (RTE) dish), or cooked for a very short time, thus the presence of pathogens on the leaves hold potentially large health risks for the consumers (Guzman-Herrador et al., 2011; Pakalniskiene et al., 2009; Pezzoli et al., 2008). As demonstrated by Gorbatsevich et al. (2013), surface contamination with *Salmonella* poses a much higher risk for human infection than internal contamination, because the internalized *Salmonella* survived less than 22 h, while *Salmonella* was inactivated at a lower rate on basil leaves on growing plants and thus remained present for at least 8 days. Kisluk et al. (2013) concluded that *Salmonella* was transferred from irrigation water (spray irrigation) to basil plants and remained detectable at 2.1 log cfu/g (detection limit 2.0 log cfu/g) on basil leaves of growing plants for 100 days after the initial inoculation with 8 log cfu/g. In addition, survival of *Salmonella* and *E. coli* O157:H7 was observed on harvested leaves stored at 4°C until the end of the shelf life (19 days post inoculation) (Hsu et al., 2006), whereas at 25°C an increase of *Salmonella* of more than 2 log units was observed after 3 days by Kisluk et al. (2013). In the present study, survival was not performed at 4°C to avoid chilling injuries (Lange & Cameron, 1994). At 7°C in the present study, both *Salmonella* and *E. coli* O157:H7 survived for one week with a reduction of 0.3 to 1.8 log units. In contrast to Kisluk et al. (2013), no growth of *Salmonella* or *E. coli* O157:H7

was observed after 3 days at 22°C. Even after 6 or 7 days (22°C) growth was rather restricted. Basil contains essential oils known to be active/inhibitory against foodborne pathogens as *E. coli* O157:H7 and *S.* Typhimurium (Elgayyar et al., 2001). Sensitivity to basil oil depend on the pathogens, but also on the serovar (Kisluk et al., 2013). Essential oils are volatile secondary metabolites of an aromatic plant's secondary metabolism and are normally produced in special cells on the leaf surface (Oussalah et al., 2007). Kisluk et al. (2013) reported that *Salmonella* was not affected by parsley oil in contrast to basil oil. In addition, Gorbatsevich et al. (2013) suggested the influence of essential oils on the survival of *Salmonella* on basil leaves as a higher decay rate on the adaxial surface (with a higher concentration of cells) was found compared to the abaxial side. Due to the limited decrease/increase in the present study, it is suggest that antimicrobial compounds do not influence survival or growth of both pathogens on basil leaves during storage, possibly by the entrapment of the essential oils in plant cells or the development of resistance to the essential oils. Moreover, the limited growth may also be explained due to the presence of high level of background microbiota (competition for space and nutrients) or the limited presence of nutrients on the leaf surface. In addition, basil leaves were inoculated by immersion and washed before storage in Kisluk et al. (2013), while in the present study basil leaves were spot inoculated and not washed, which may explain why less increase of both pathogens on basil leaves was observed at 22°C.

Leafy greens have been studied less than aromatic plants as basil for antimicrobial properties. However, Al Nomaani (2013) reported the presence of some antimicrobial components (e.g. linalool) in lettuce. The highest decrease was observed for both pathogens on lamb lettuce, while the highest increase on fresh-cut iceberg lettuce and butterhead lettuce. In general, pathogens will survive but not grow on the uninjured outer surface of fresh fruits and vegetables by among other the plant natural barriers, e.g. wax layers and cell walls (Harris et al., 2003). As fresh-cut iceberg lettuce and butterhead lettuce leaves have relative big cut edges compared to the other leafy greens, more cell exudates are released which provides nutrients for microbial growth (Delaquis et al., 2007). However, using spot inoculation minimalizes the chance that the inoculum is exposed to cell exudates. The latter may also explain the rather limited growth of both pathogens on iceberg lettuce and butterhead lettuce. However, both Francis & O'Beirne (2001) and Koseki & Isobe (2005) used

spot inoculation, but a higher increase of *E. coli* O157:H7 and/or *Salmonella* was reported when stored at temperatures higher than 7°C. But in previous mentioned study, the sample was shaken after inoculation whereby the chance is again increased that the inoculum comes into contact with cell exudates. Also Chang & Fang (2007) reported a higher increase after 3 days storage at 22°C. The latter may be explained by the higher inoculum volume used, which increases the water availability and humidity. Koseki & Isobe (2005) created a predictive model which described the behavior of *E. coli* O157:H7 and *Salmonella* whereby the model was based on survival studies on fresh-cut iceberg lettuce. The model has been applied for example in a quantitative risk assessment of the generic group of leafy greens (Franz et al., 2010). But as shown in the present study, as the survival and/or growth of *E. coli* O157:H7 and *Salmonella* can be different for various types of leafy greens, in particular when (baby) leaves (with less cut surfaces and exudates) are implicated, the use of the by Koseki and Isobe published model can overestimate growth of these pathogens on leafy greens other than fresh-cut iceberg lettuce.

Strawberries were shown to be less supportive for survival and induce die-off of the pathogens throughout the storage period which gradual progressed below the detection limit of enumeration. Survival of *Salmonella* and *E. coli* O157:H7 on basil leaves during storage was observed for at least one week at all temperatures. The highest increase of pathogens was observed at fresh-cut iceberg lettuce and butterhead lettuce when stored at 15°C or 22°C (probably due to the presence of exudates and lack of natural antimicrobial substances in these lettuce varieties). However these product are mostly stored at cold temperatures where no increase was observed. Furthermore, washing by the consumers has only a limited effect (max. 0.9 log units reduction) on pathogens on strawberries (Hung et al., 2010; Rodgers et al., 2004; Yu et al., 2001). For basil, no studies were yet performed relating to the effect of washing at consumers' houses, but from Keskinen & Annous (2011) and Sengun (2013) it could be derived that washing lettuce or parsley with water has only a limited effect on the removal of *E. coli* O157:H7 and *Salmonella*. In conclusion, once contaminated with *Salmonella* or *E. coli* O157:H7, strawberries and basil may pose a considerable risk to human health as both pathogens can be highly infectious (Harris et al., 2003; Teunis et al., 2004; Teunis et al., 1996). Because of the lack of any pathogen

inactivation during storage and preparation, prevention of contamination through good agricultural practices should be strived for.

CHAPTER 4: Microbial safety and sanitary quality of strawberry primary production in Belgium: risk factors for *Salmonella* and Shiga toxin-producing *Escherichia coli* contamination.

Redrafted from:

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ABSTRACT

Strawberries are an important fruit in Belgium both in production and consumption, but little information is available about the presence of *Salmonella* and STEC in these berries, the risk factors in agricultural production and possible specific mitigation options. In 2012, a survey was undertaken of three soil and three soilless cultivation farms in Belgium. No *Salmonella* was isolated. No STEC was detected in the strawberry samples (0 out of 72), but STEC was detected by PCR in 11 out of 78 irrigation water and 2 out of 24 substrate samples. Culture isolates were obtained for 2 out of 11 PCR positive irrigation water samples and 2 out of 2 PCR STEC positive substrate samples. Multivariate analysis revealed elevated generic *E. coli* numbers (odds ratio (OR) for 1 log increase being 4.6) as the most important risk factor for positive STEC PCR signals, together with the berry picking season (highest in summer, followed by spring (OR = 0.1) and fall (OR = 0.05)). The presence of generic *E. coli* was determined by the sample type, the season, the irrigation water type and application of water treatment. The likelihood of detection of generic *E. coli* in the irrigation water (≥ 1 cfu per 100 ml) was mainly influenced by the type of irrigation water (collected rainfall water stored in ponds was more often contaminated than ground water pumped from boreholes (OR = 5.9)) and the lack of prior treatment (untreated water versus water subjected to sand filtration prior to use (OR = 19.2)). A follow-up study in farm 1 in 2013 indicated cattle as the most likely source of STEC contamination of irrigation water.

4.1 Introduction

This survey provides more data about the presence and levels of generic *E. coli* and the prevalence of *Salmonella* and STEC on strawberries to facilitate future risk assessments and the setting of criteria. Moreover, the safety and sanitary quality of strawberries production environment, in both soil and soilless cultivations types, was also investigated into more detail through sampling of the production environment (water, soil/substrate) and workers' hands to determine the environmental pressure and risk factors for the contamination with pathogens in the whole strawberry production environment.

4.2 Material en methods

4.2.1 Sampling

Six farms which produces strawberries (cultivar Elsanta) in Flanders, Belgium, were sampled four times during the fruiting season and thus strawberries picking period in 2012, i.e. from April to December. Three farms used cultivation in soil (unprotected in open field and/or protected in plastic tunnels) and three used soilless cultivation (in greenhouses and/or in plastic tunnels in substrate, e.g. peat or coconut fibers) (Table 7). Information about the farm, the production system and the environment was collected using a questionnaire and visit to each farm (ANNEX 1). Climatic data, namely the average daily temperature (°C) and daily precipitation (> 1 mm), were retrieved from http://www.worldweatheronline.com/ for the exact location of the farms with the exception of farm 2, for which the nearest available point was at 7 km distance. Each visit, samples were taken of strawberries (n = 9/visit), irrigation water (n = 2 or 4/visit; if possible 2 samples of the source of the irrigation water and 2 samples at the actual starting point of the irrigation network i.e. the intermediate reservoir), soil (n = 6/visit; substrate in case of soilless production and a swab of the black plastic foil covering the soil in (open) field production) and hands of the pickers (n = 1 to 5/visit) (Figure 12). The number of hands sampled depended on the number of workers present on the farm: < 5 = 1 sample; 5-10 = 3 samples; ≥ 10 = 5 samples. The whole surfaces of hands (approx. 25 cm^2) were sampled by rayon swabs (Fortuna Scientific, Singapore; filled

with 5 ml Physiological Peptone Salt solution (PPS; 8.5 g/l NaCl (Fluka, Switzerland) and 1 g/l neutralized bacteriological Peptone (Oxoid, UK)).

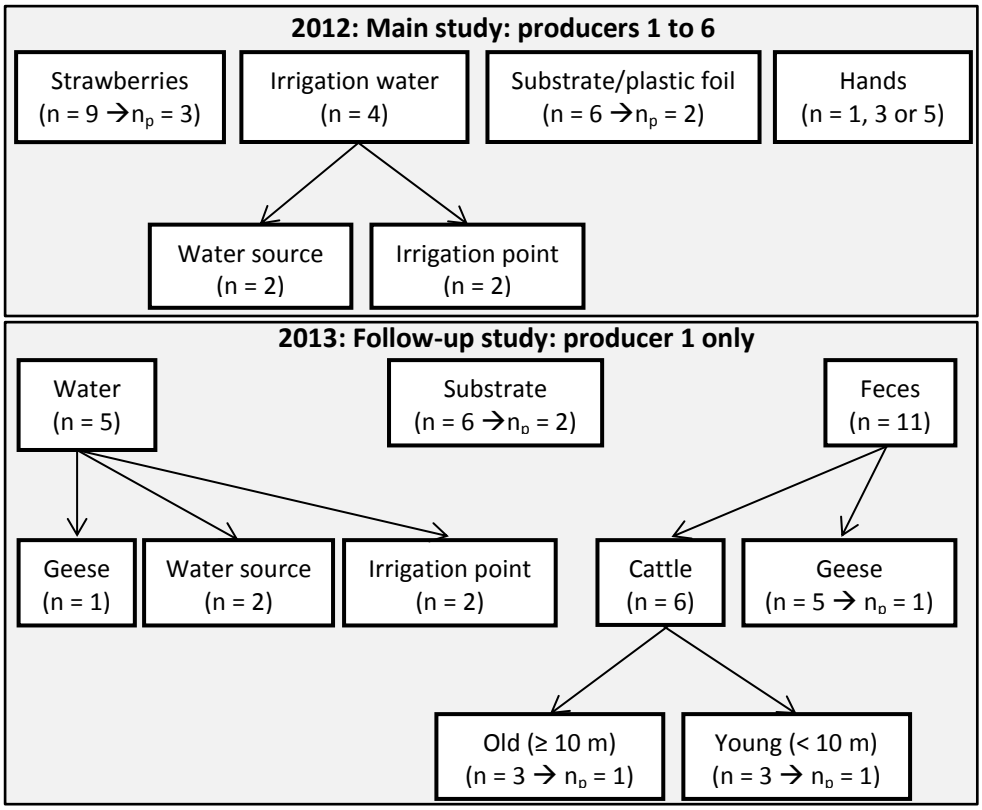

Figure 12: Samples taken per visit (n) and amount of samples analyzed after pooling (n_p) during the main study in 2012 (4 visits at 6 producers) and the follow-up study in 2013 (12 visits at producer 1).

Strawberry samples consisted of 10 whole berries per sample which were picked with gloves and collected in Twirl'm bags (Labplas, Canada) (Figure 13). In the laboratory, strawberry samples were cut into smaller particles with a blender and subsamples of 20 g were pooled per 3 samples into one sample of 60 g. Substrate samples of one handful were collected in Twirl'm bags and in the laboratory 20 g of 3 samples was pooled into one of 60 g. The black plastic foil covering the soil in open fields was sampled by swabbing 1000 cm² with a sponge-stick with 10 ml Buffered Peptone Water (BPW) (3M, St. Paul, USA). Three sponge swabs were pooled to one sample. Approximately 1.5 l water per sample was collected in sterile bottles. Water samples were taken after letting the water run for 1 minute and the temperature of the sampled water was immediately measured. In the laboratory, 1 l and 100 ml of a water sample were filtered on a cellulose nitrate filter (pore size 0.45 µm, Sartorius Stedim Biotech GmbH, Germany) for pathogen detection and *E. coli* enumeration,

respectively. If necessary, more filters were used to filter the required water volume when clogging by small particles occurred.

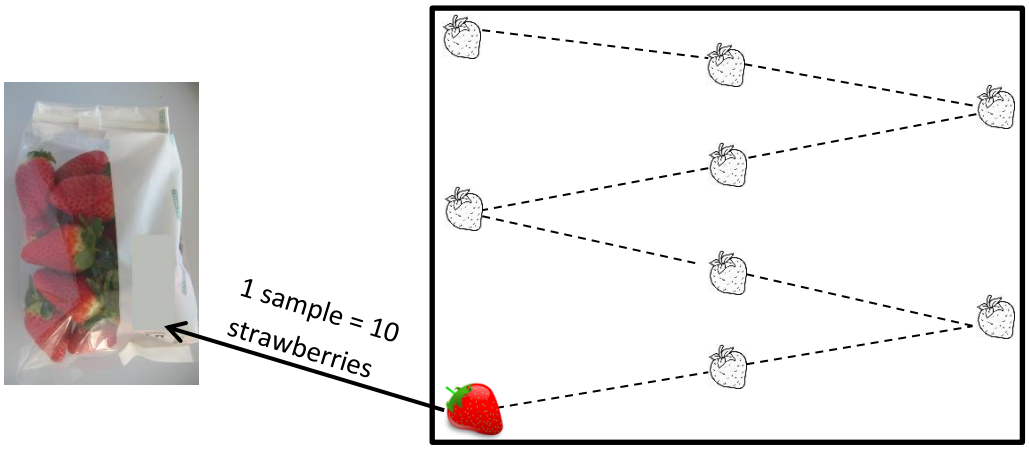

Figure 13: Overview of the distribution of the strawberry samples taken on the strawberry field during one visit and the composition of one strawberry sample (1 sample = 10 strawberries)

Subsequent to the 2012 survey including 6 farms, a follow-up study was performed at one of the farms in the fruiting season of 2013, using a more frequent monitoring plan with sampling focused in particular on the strawberries' production environment and bi-weekly sampling during 12 weeks (May 2013 until October 2013). Samples were taken from again the irrigation water (n = 4/visit) and the substrate (n = 6/visit) but also fecal samples of the beef cattle on the farm (n = 6/visit) and of the geese present near the irrigation water pond (n = 5/visit) (Figure 12). Also, every visit a water sample was taken from the small bathing pond of the geese (n = 1/visit) because it was located next to the irrigation water pond and run-over occurred during rainfall. In case of the beef cattle, 3 samples of older cattle (> 10 months up to 3 years age) and 3 samples of the younger cattle (< 10 months) were taken. Irrigation water and substrate samples were collected as described above. Pond water of the geese was analyzed for pathogens by filtration of 100 ml instead of 1 l (as higher volumes were not possible due to clogging of the filter) and no *E. coli* was enumerated. Cattle feces (between 14 and 54 g) were collected by walking around in an 8-shaped track in the stable wearing one pair of liquid absorbing overshoes (Kolmi, Saint Bathélémy d'Anjou, France) as described in (Cobbaut et al., 2008). Overshoes were collected in filter stomacher bags (FBAG-04, Novolab, Belgium) and pooled per 3 samples after enrichment. Five gram of geese droppings was collected in Twirl'em bags and 5 samples were pooled to one sample of 25 g.

Table 7: Overview of the characteristics of the six strawberry farms sampled in 2012.

Producer	1	2	3	4	5	6
Cultivation type	Soilless (greenhouse and plastic tunnel)	Soil (plastic tunnel)	Soil (plastic tunnel and open air)	Soilless (greenhouse)	Soilless (greenhouse)	Soil (plastic tunnel and open air)
Irrigation water source	Rain water	Rain water	Ground and rain water	Rain water	Rain water	Ground water
Irrigation water storage	Pond with plastic foil	Pond	Borehole and pond	Pond	Raised pond with plastic foil	Borehole
Irrigation way	Drip	Drip	Drip	Drip	Drip	Drip
Irrigation water treatment	None	None	None	Sand filtration*	Sand filtration	None
Fertilizer type	Mineral	Mineral	Mineral and composted vegetable material	Mineral	Mineral	Mineral and composted cattle manure
Farm animals present	Cattle and geese	Cattle	None	None	None	Cattle and sheep
Toilet distance from field	50 m	100 m	150 m	50 m	50 m	150 m
Sampling period	May - November	May - June	May - August	April - December	May - December	May - June
Number of visits	4	4	4	4	4	4
Average daily temperature						
Median (°C)	16.8	15.0	16.2	9.5	11.2	14.8
Range (minimum - maximum)	10.0 - 19.5	8.0 - 16.5	16.0 - 16.5	0.5 - 10.0	4.5 - 19.5	8.0 - 16.5
Daily precipitation (mm)						
Days without precipitation	1/4	1/4	2/4	0/4	1/4	2/4
Range (minimum - maximum)	1.3 - 13.5	9.4 - 22.3	2.0 - 3.8	1.1 - 8.5	1.1 - 9.1	8.5 - 10.7
Flooding	0/4	0/4	1/4	0/4	0/4	0/4
Irrigation water temperature (°C)						
Median	19.6	16.5	16.2	13.8	14.8	17.2
Range (minimum - maximum)	13.6 - 25.4	14.1 - 19.4	15.0 - 23.1	12.0 - 15.0	6.1 - 19.3	15.2 - 22.0

*In addition, drain water is reused after reconditioning by sand filtration and UV irradiation

All samples were transported on ice in a cool box, stored at 4 °C and analyzed within 24 h.

4.2.2 Microbiological analysis

Samples of strawberries, water, substrate and plastic foil on the soil were analyzed for the presence/absence of *Salmonella* and STEC per 60 g for strawberries and substrate, per 3000 cm² for the plastic foil, per 1 l for irrigation water and per 100 ml pond water and the enumeration of *E. coli* as hygienic indicator. The farm workers' hands were analyzed for the amount of *E. coli* and Enterobacteriaceae. Cattle and geese feces were only analyzed for the presence/absence of *Salmonella* and STEC.

4.2.2.1 E. coli

The Petrifilm Select *E. coli* (LED techno, Belgium) was used for the enumeration of *E. coli* in samples of strawberries, substrate, foil and hands by applying 1 ml of the appropriate tenfold dilution in BPW followed by incubation at 37°C for 24 h ± 2 h. Filters from water samples were incubated on Rapid'*E. coli*2 (REC2; Bio - Rad, France) plates at 42°C ± 1°C for 24 h ± 3 h for the enumeration of *E. coli*.

4.2.2.2 Enterobacteriaceae

Enterobacteriaceae in the swab samples of hands (1 ml) were enumerated on pour plates of Violet Red Bile Glucose (VRBG) agar with an overlayer after incubation at 37°C for 24 h ± 2 h.

4.2.2.3 *Salmonella* and Shiga toxin-producing *E. coli* (STEC)

Samples for pathogen detection were enriched in a filter stomacher bag fivefold diluted (tenfold diluted for the geese feces and approx. 300 ml added to an overshoe) with BPW at 37°C for 18 – 24 h. In case of water, filters were enriched in 150 ml BPW. *Salmonella* and STEC were detected by real-time PCR of the *iroB* gene for *Salmonella* and the *stx1*, *stx2*, *eae* and *aggR* genes for STEC with the GeneDisc plate *Salmonella* & aggregative *E. coli* as described in Beutin et al. (2009). In short, fifty microliter of the enriched sample was put in a lysis tube for the DNA extraction by heating (10 min, 100°C) followed by centrifugation (2 min, 10,000 *x g*). Subsequently, the supernatants was diluted, using the dilution buffer (Extraction Pack Food 1, Pall Technology) to prevent inhibition of the PCR reaction, as observed in a preliminary study (strawberries, water and feces: ½ dilution; substrate and

soil: ¼ dilution). *Salmonella* PCR positive samples were only considered positive after culture confirmation as described in ISO 6579:2002, because false-positive results were obtained during our prior study to validate the selected methods on fresh produce samples (CHAPTER 2). Briefly, non-selective enrichment in 10 ml BPW at 37°C for 18 – 24 h was followed by a selective enrichment in 10 ml Rappaport-Vassiliadis Soya Broth (RVS; Biomérieux, Belgium) at 42°C and in 10 ml Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn; Biomérieux) at 37°C. After 24 ± 3 h incubation, samples were plated on Xylose-Lysine-Deoxycholate agar (XLD; Oxoid) and *Salmonella Brilliance* (Oxoid) and typical colonies were confirmed by Crystal ID E/NF (BD, Belgium). STEC is defined by the Shiga toxin genes *stx1* and/or *stx2* and in our study the combination of *stx1/2* with adhesion genes *eae* or *aggR* was determined due to the enhanced pathogenic potential of adhesive STEC (EFSA, 2013c; Tozzi et al., 2003). After positive PCR results, culture confirmation for *E. coli* O157 was performed according to ISO 16654:2001. Briefly, immunomagnetic separation (IMS) with Dynabeads anti-*E. coli* O157 (Invitrogen Dynal AS, Norway) was followed by plating on Cefixime Tellurite Sorbitol MacConkey agar (CT-SMAC; Oxoid) and on SMAC with chromogen 5-bromo-4-chloro-3-indolyl-b-D-glucuronide *(*BCIG). Moreover, PCR positive samples were further analyzed for the serotypes O26, O103, O111 and O145 and for the *eae* variants β, θ, ϒ and ε by PCR (the GeneDisc STEC identification plate and the GeneDisc STEC plus plate (Pall Technologies), respectively), again because these serotypes have increased pathogenic potential (EFSA, 2013c; Posse et al., 2007). Specific STEC serotypes are associated with specific *eae* variants, namely STEC O157 and O145 possess the ϒ variant, O111 harbors the ε or ϒ variant, O103 has the θ or ϒ variant and O26 the β variant (Posse et al., 2007). This way, the (im)possibility of the *stx* and *eae* virulence factors originating from one *E. coli* strain is gauged by the match of the *eae* variant and the serotype detected by PCR. Culture confirmation was also performed for non-O157 *E. coli* by streaking samples on CHROMagar STEC (CHROMagar Microbiology, France) and/or CHROM ID STEC (Biomérieux). In addition, both the enriched sample as isolated *E. coli* presumptive colonies (n = 3/sample) were further sent off for potential STEC isolation or confirmation of STEC serotypes by the national expert lab on STEC (the Lab of Hygiene and Technology, Faculty of Veterinary Medicine, Ghent University). In this lab, samples were plated on CT- SMAC for STEC O157 and on MAC for non-O157 STEC (as well the obtained presumptive isolates as the enriched sample). STEC presumptive colonies obtained from CT-SMAC and/or MAC were further

analyzed by multiplex PCR (*stx1*, *stx2* and *eae*). If virulence genes were detected, isolates were analyzed for the serotype by PCR.

4.2.3 Statistical analysis

The 95 % confidence interval (C.I.) on prevalence estimates was calculated by the Wilson score method without continuity correction (Wilson, 1927). Ordinal classes were defined for the enumeration data of generic *E. coli* (Table 8), because *E. coli* was undetected in many samples and depending on the sample type and microbiological analysis method different detection limits were obtained.

Table 8: Definition of classes to group the generic *E. coli* enumeration data

***E. coli* class**	**Water**	**Strawberry and substrate**	**Hands**	**Plastic foil on soil**
1[a]	< 0.0 log cfu/100 ml	< 1.0 log cfu/g	< 0.7 log cfu/ 25 cm²	< 2.6 log cfu/ 3000 cm²
2	≥ 0.0 and < 1.0 log cfu/100 ml	≥ 1.0 log cfu/g	≥ 0.7 log cfu/ 25 cm²	≥ 2.6 log cfu/ 3000 cm²
3	≥ 1.0 and < 2.0 log cfu/100 ml	NA	NA	NA
4	≥ 2.0 and < 3.0 log cfu/100 ml	NA	NA	NA
5	≥ 3.0 log cfu/100 ml	NA	NA	NA

[a]: undetected; NA = Less than five samples would be present in higher classes with tenfold differences in concentrations, so they were merged to the previous class.

Results were processed with SPSS version 21 at a significance level of 95 % (p = 0.050). In case of multiple pairwise comparisons, Bonferroni correction was applied to control the family-wise error rate at 5 %. The chi-squared test of independence (likelihood ratio) and the Kendall's tau-c test were used to investigate possible relations between STEC and *E. coli,* respectively, and several categorical agro-technical factors, such as the production system (soil versus soilless production), and source of irrigation water (collected rainfall water in ponds versus freshly pumped up borehole water), etc. Continuous variables (i.e. average daily temperature, daily precipitation and water temperature) were not normally distributed (Kolmogorov-Smirnov test, p < 0,001) so Mann-Whitney U tests were used to assess potential differences in the presence/absence of STEC by PCR detection and Spearman's rho correlation coefficient for potential correlation with generic *E. coli* concentrations. Multiple

logistic regression was applied to identify the factors of influence considered simultaneously, their relative importance and possible interactions on the PCR detection of STEC. Backward likelihood ratio model selection was performed on all significant factors for significant main effects and all possible interactions between the obtained effects were checked one-by-one by addition to the final model. Due to the large number of samples in which generic *E. coli* was not detected (194/255 = 76 %), multiple logistic regression was also applied for *E. coli* presence, rather than a generalized linear model with Poisson distribution on the *E. coli* counts.

4.3 Results

4.3.1 Main study: sanitary quality of Belgian strawberry production

In 2012, no *Salmonella* and no STEC were detected on strawberries (0/72; 95 % C.I.: 0.0 – 5.1 %), whereas *E. coli* was enumerated on 2/72 strawberry samples (1.0 log cfu/g and 3.0 log cfu/g), both sampled in a company performing cultivation in soil during 2 different visits. In water no *Salmonella* was detected (0/78; 95 % C.I.: 0.0 – 4.7 %), whereas STEC was detected by PCR in 11/78 irrigation water samples (Table 9).

Table 9: Summary of the microbiological parameters of the irrigation water.

Producer	1	2	3	4	5	6
Generic *E. coli*						
Presence (per 100 mL)	16/16	8/8	1/6 (borehole); 2/2 (pond)	5/14	9/16	3/16
Mean (log CFU/100 mL) ± standard deviation	1.6 ± 0.8	3.1 ± 0.9	0.0 ± 0.0 (borehole); 1.8 ± 0.0 (pond)	1.4 ± 0.9	0.6 ± 0.5	0.0 ± 0.0
STEC						
Presence of *stx1/2* gene(s)	5/16	5/8	0/8	1/14	0/16	0/16
Presence of *stx1/2* and *eae* genes	5/16	5/8	0/8	1/14	0/16	0/16
Presence of *stx1/2* and *eae* genes, serotypes O26, O103, O111 and O145	4/16	3/8	0/8	1/14	0/16	0/16
Culture isolate	2/16	0/8	0/8	0/14	0/16	0/16

The *stx* toxin genes were always detected simultaneously with the adhesion *eae* gene, namely *stx1* and *eae* (4/11), *stx2* and *eae* (6/11) and *stx1* and *stx2* and *eae* (1/11) (Table 10).

It should be noted that the *eae* gene (without the presence of *stx1/2* genes) was detected in 2/72 strawberry samples, 22/78 irrigation water samples, 5/24 substrate samples and on 6/24 plastic covers, while the *aggR* gene was never detected (neither alone or in combination with *stx1* and *stx2* genes).

Further PCR analysis showed the corresponding combination of serotype and *eae* variant for 8 of these 11 samples. Culture isolation was successful for 2 of the 11 PCR positive water samples and yielded a STEC O26 strain which possessed *stx1* and *eae*. *E. coli* was enumerated in 44/78 irrigation water samples ranging from 1 cfu/100 ml to 15,849 cfu/100 ml and respectively 10/44 and 6/44 samples exceeded a value of 100 and of 1000 cfu/100 ml. All 6 samples exceeding 1000 cfu per 100 ml were obtained in one production site from a pond located next to a pasture. As for strawberries and water, in the 2012 survey no *Salmonella* was detected in the sampled substrate (0/24; 95 % CI: 0.0 – 13.8 %) nor on the plastic foil covering the soil (0/24; 95 % CI: 0.0 – 13.8 %), although STEC was detected in 2/24 (95 % CI: 2.3 – 25.8 %) substrate samples but not on the plastic cover (0/24; 95 % CI: 0.0 – 13.8 %). Both substrate samples showed the presence of the corresponding serotype and *eae* variant combination and a STEC O26 isolate was obtained from both samples (Table 10). *E. coli* could be enumerated in 7/24 substrate samples (mean concentration of the positive samples 1.8 log cfu/g ± standard deviation 1.3 log cfu/g) and on 4/24 plastic covers (3.0 ± 0.5 log cfu/3000 cm²). *E. coli* and Enterobacteriaceae were enumerated on 4/57 (1.8 ± 1.2 log cfu/25 cm²) and 33/57 (1.9 ± 1.1 log cfu/25 cm²) of the hands, respectively. The presence of *E. coli* on the hands indicates fecal contamination and the presence of Enterobacteriaceae may indicate lack of sufficient and effective hand washing practices.

Table 10: Overview of STEC PCR-positive samples during the sampling in 2012 and 2013, supplemented with the *eae* variant (*stx* genes were always detected simultaneously with *eae*), the serotype (PCR detected), the result of culture confirmation and the enumeration of generic *E. coli*.

Producer	Sample	Date	GeneDisc (PCR)					Culture confirmation	Generic *E. coli* (cfu/100 ml or g)[e]
			stx1	*stx2*	*eae*	serotype	combination *eae* - serotype*		
2	Water[a]	6/06/2012		x	β, θ, ε	O26, O103, O145	O26, O103	No	$1{,}1 \times 10^{3}$
2	Water[a]	18/06/2012		x	β	O26, O103, O145	O26	No	$1{,}2 \times 10^{3}$
2	Water[a]	31/05/2012		x	β, θ	O26, O103, O145	O26, O103	No	$1{,}4 \times 10^{4}$
2	Water[a]	15/05/2012		x	θ	O145, O26	No	No	$6{,}0 \times 10^{1}$
2	Water[a]	15/05/2012		x	θ	O145, O26	No	No	$5{,}8 \times 10^{1}$
4	Water[b]	8/10/2012	x	x	β, θ, ε	O26, O103, O145	O26, O103	No	$1{,}6 \times 10^{2}$
1	Water[a]	4/07/2012		x	-	O145, O26	No	No	$2{,}1 \times 10^{1}$
1	Water[a]	14/09/2012	x		β, θ, ε, ϒ	O26, O103, O145	O26, O103, O145	Yes (O26[f])	$1{,}7 \times 10^{2}$
1	Water[b]	14/09/2012	x		β, ε, ϒ	O26, O145	O26, O145	Yes (O26[f])	$1{,}6 \times 10^{2}$
1	Water[b]	14/09/2012	x		β, θ, ϒ	O26, O103, O145	O26, O103, O145	No	$1{,}6 \times 10^{2}$
1	Water[a]	14/09/2012	x		β, ε, ϒ	O26, O145	O26, O145	No	$1{,}9 \times 10^{2}$
1	Substrate	14/09/2012	x		β	O26, O103, O145	O26	Yes (O26[f])	$2{,}0 \times 10^{1}$
1	Substrate	14/09/2012	x	x	β, ϒ	O26, O145	O26, O145	Yes (O26[f])	$2{,}0 \times 10^{1}$
1	Water[b]	15/05/2013	x		β	O26, O145	O26	No	$1{,}5 \times 10^{2}$
1	Water[a]	15/05/2013	x		β, θ	O26, O145	O26	No	$8{,}6 \times 10^{1}$
1	Water[b]	21/08/2013	x		-	-	No	No	$1{,}1 \times 10^{1}$
1	Water[a]	18/09/2013		x	β, θ	O103, O145	O103	No	$2{,}1 \times 10^{2}$
1	Water[b]	15/10/2013		x	β, θ, ϒ	O26, O103, O145	O26, O103, O145	No	$1{,}9 \times 10^{3}$
1	Water[a]	15/10/2013		x	β, ϒ	O26, O145	O26, O145	No	$2{,}3 \times 10^{2}$
1	Water[a]	15/10/2013		x	β, ϒ	O26, O145	O26, O145	No	$2{,}2 \times 10^{2}$
1	Substrate	15/05/2013	x		-	O26	No	No	$1{,}1 \times 10^{1}$
1	Geese feces	29/05/2013	x	x	-	-	No	No	ND
1	Cattle feces[c]	15/05/2013	x	x	β, θ	O26, O145	O26	No	ND
1	Cattle feces[d]	15/05/2013	x	x	β, θ	O26, O145	O26	No	ND

(Continue of Table 10)

Producer	Sample	Date	GeneDisc (PCR)					Culture confirmation	Generic *E. coli* (cfu/100 ml or g)[e]
			stx1	*stx2*	*eae*	serotype	combination *eae* - serotype*		
1	Cattle feces[c]	29/05/2013	x	x	β, θ	O26, O145	O26	No	ND
1	Cattle feces[d]	29/05/2013	x	x	β, θ, ε	O26, O145, O103, O111	O26, O103, O111	No	ND
1	Cattle feces[c]	12/06/2013	x	x	θ, β	O145	No	No	ND
1	Cattle feces[d]	12/06/2013	x	x	β, θ, ϒ	O26, O145, O103	O26, O103, O145	No	ND
1	Cattle feces[c]	26/06/2013	x	x	θ, ε	O145, O103	O103	No	ND
1	Cattle feces[d]	26/06/2013	x	x	β, θ, ε, ϒ	O26, O145, O103	O26, O103, O145	No	ND
1	Cattle feces[c]	8/07/2013	x	x	β, θ, ε	O145, O103	O103	No	ND
1	Cattle feces[d]	8/07/2013	x	x	β, θ, ε, ϒ	O145, O103	O103, O145	No	ND
1	Cattle feces[c]	24/07/2013	x	x	β, θ, ε	O145, O103	O103	No	ND
1	Cattle feces[d]	24/07/2013	x	x	β, θ, ε, ϒ	O145, O103	O103, O145	No	ND
1	Cattle feces[c]	7/08/2013	x	x	β, θ, ε	O145, O103	O103	No	ND
1	Cattle feces[d]	7/08/2013	x	x	β, θ, ε, ϒ	O145, O103	O103, O145	No	ND
1	Cattle feces[c]	21/08/2013	x	x	β, θ, ε	O145, O103	O103	No	ND
1	Cattle feces[d]	21/08/2013	x	x	β, θ, ε, ϒ	O145, O103	O103, O145	No	ND
1	Cattle feces[c]	4/09/2013	x	x	β, θ, ε	O145, O103	O103	No	ND
1	Cattle feces[d]	4/09/2013	x	x	β, θ, ε, ϒ	O26, O145, O103	O26, O103, O145	No	ND
1	Cattle feces[c]	18/09/2013	x	x	θ, ϒ	O145, O103	O103, O145	No	ND
1	Cattle feces[d]	18/09/2013	x	x	β, θ, ε	O145, O103	O103	No	ND
1	Cattle feces[c]	1/10/2013	x	x	β, θ, ϒ	O145, O103	O103, O145	No	ND
1	Cattle feces[d]	1/10/2013	x	x	β, θ, ε	O145, O103	O103	No	ND
1	Cattle feces[c]	15/10/2013	x	x	β, ε, ϒ	O145, O103	O103, O145	No	ND
1	Cattle feces[d]	15/10/2013	x	x	β, θ, ε	O145, O103	O103	No	ND

*Serotypes for which the corresponding *eae* variant was detected; [a]Irrigation point; [b]Source; [c]Feces from cattle ≥ 10 months old; [d]Feces from cattle < 10 months old; [e]Depending on matrix: water (cfu/100 ml), substrate (cfu/g); [f]STEC O26 *stx1*+ & *eae*+ (PCR confirmed); - = No *eae* variant or serotype was identified; ND = not determined.

4.3.2 Risk factors for the presence of STEC and the fecal indicator *E. coli*

The presence of STEC was significantly correlated with the *E. coli* class (= level of *E. coli*), which means that samples positive for STEC were associated with a higher *E. coli* class and thus had a higher average *E. coli* count (Figure 14).

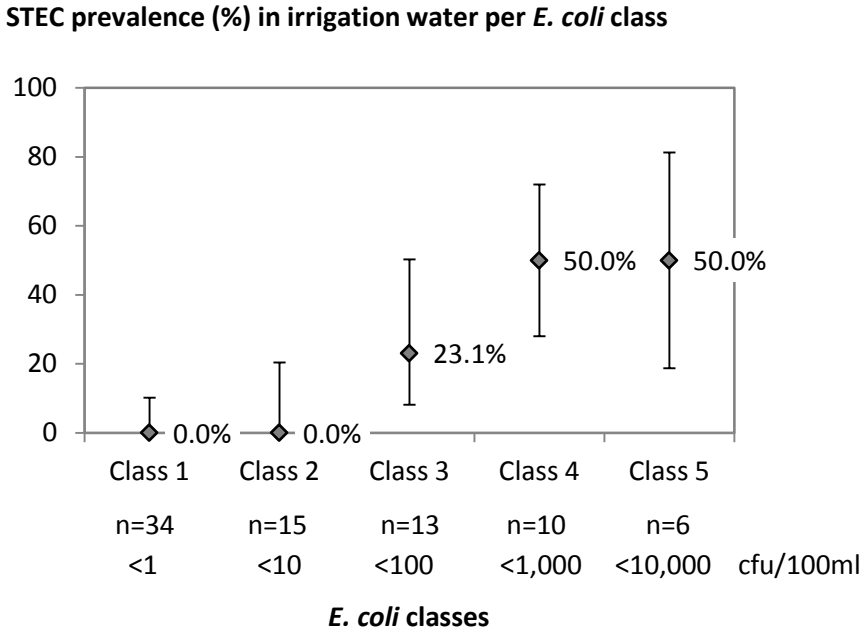

Figure 14: Correlation between the presence of STEC (detection by PCR) and the generic *E. coli* class (see concentrations associated with the classes in Table 8) in irrigation water samples (n = 78; 2012).

Following factors were significantly correlated with the STEC prevalence and the generic *E. coli* counts in univariable statistical analysis (meaning without considering the other factors): i) the sample type, (ii) the producer, (iii) the presence of farm animals, (iv) the irrigation water type, (v) treatment of the irrigation water and (vi) the time of the year (month/season) (Table 11).

Cultivation type (soil or soilless cultures) was not of significant influence on the prevalence of STEC and the *E. coli* counts, only the Enterobacteriaceae count was higher on the hands of workers in soil cultivation in comparison with soilless cultivation (Figure 15). Interestingly, there was significant variation in STEC and *E. coli* between different farms, suggesting that there are specific farm factors influencing the microbiological sanitary quality and safety. Seasonal differences were observed with the highest prevalence of STEC (over all sample

types) in September and the highest generic *E. coli* counts in September and October. In Figure 16 and Figure 17 the seasonal differences are presented for irrigation water.

Table 11: Significance of potential risk factors on STEC PCR detection and generic *E. coli* concentrations by univariable statistical analysis (2012).

Parameter	STEC	*E. coli*
Cultivation type	- (p = 0.547)	- (p = 0.099)
Producer	+ (p < 0.001)	+ (p < 0.001)
Sample type	+ (p < 0.001)	+ (p < 0.001)
Month	+ (p < 0.001)	+ (p = 0.048)
E. coli	+ (p < 0.001)	NA
Presence of farm animals	+ (p = 0.001)	+ (p = 0.003)
Irrigation water type	+ (p = 0.001)	+ (p = 0.001)
Water treatment	+ (p = 0.016)	+ (p = 0.010)
Toilet distance	- (p = 0.639)	- (p = 0.857)
Average daily temperature	- (p = 0.783)	- (p = 0.601)
Daily precipitation	- (p = 0.836)	- (p = 0.870)
Flooding	- (p = 0.325)	- (p = 0.334)
Water temperature	- (p = 0.403)	- (p = 0.515)

NA = Not applicable

The presence of farm animals mean that cattle is being reared professionally by the producers and/or that cows or sheep are grazing on the adjacent field(s) to the strawberry production field. The presence of farm animals significantly increased the general prevalence of STEC from 1.0 % (1/98; 95 % C.I.: 0.2 – 5.6 %) to 12.0 % (12/100; 95 % C.I.: 7.0 – 19.8 %) and that of generic *E. coli* from 17.4 % (24/138; 95 % C.I.: 12.0 – 24.6 %) to 31.6 % (37/117; 95 % C.I.: 23.9 – 40.5 %).

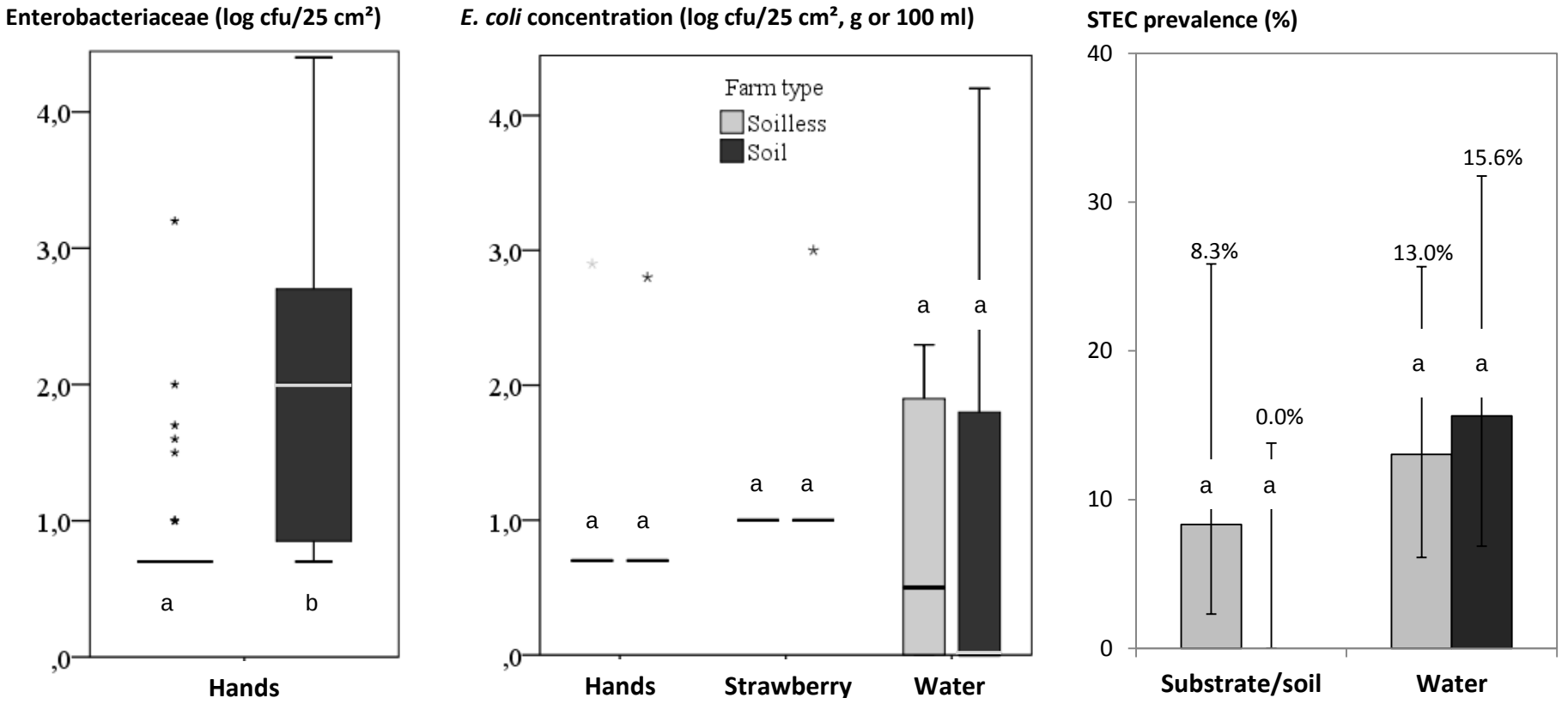

Figure 15: Boxplots show the concentrations of Enterobacteriaceae and generic *E. coli* and bars show the STEC prevalence (%; error bars present the 95 % CI) in soilless cultivation (light grey boxplots and bars) and in soil cultivation (dark grey boxplots and bars) (2012). The bottom and the top of the box of the boxplot are the 25th and 75th percentile, while the line in the box is median. The end of the whiskers are the minimum and maximum, which are not outliers or extremes (⋆).Different letters indicate statistically significant differences for the microbial parameter within one sample type.

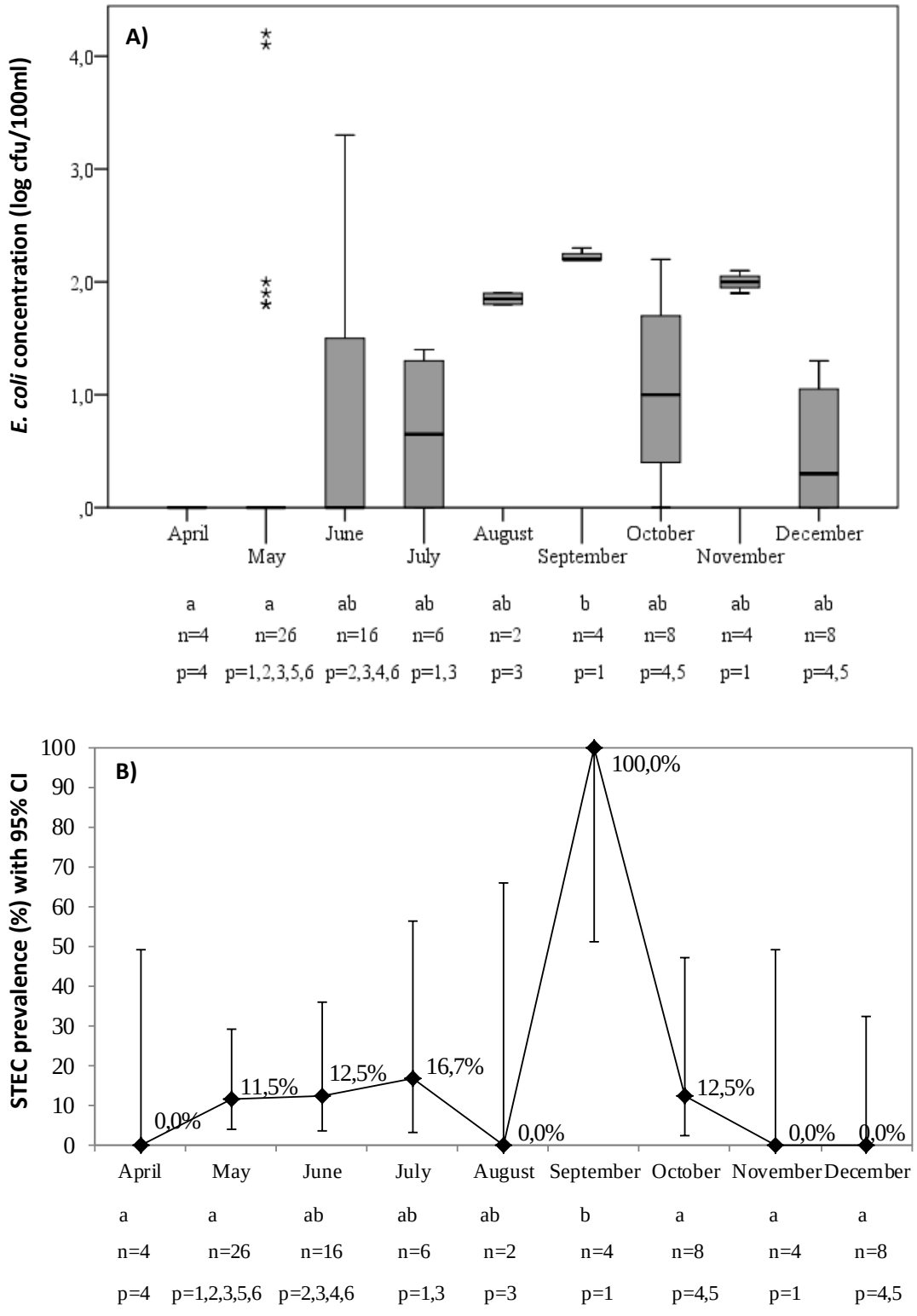

Figure 16: Boxplots showing the monthly variation in generic *E. coli* concentrations (log cfu/100 ml) (A) and STEC prevalence (%) in irrigation water during the different sampling months with error bars indicating the 95 % C.I. on the prevalence estimate (B) of all six producers in 2012. The bottom and the top of the box of the boxplot are the 25th and 75th percentile, while the line in the box is median. The end of the whiskers are the minimum and maximum, which are not outliers or extremes (★). Different letters indicate statistically significant differences between months, n = the total number of samples and p = which producers were sampled.

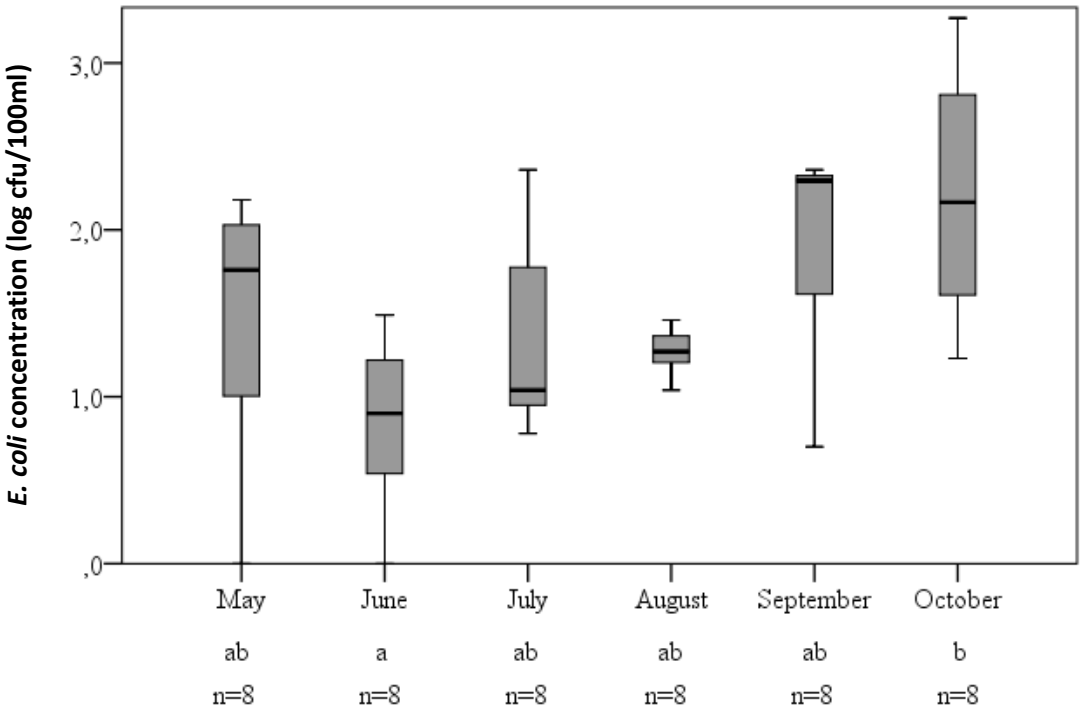

Figure 17: Boxplots showing the monthly variation in generic *E. coli* concentrations (log cfu/100 ml) in irrigation taken at producer 1 in 2013. The bottom and the top of the box of the boxplot are the 25th and 75th percentile, while the line in the box is median. The end of the whiskers are the minimum and maximum, which are not outliers or extremes (★). Different letters indicate statistically significant differences between months, n = the total number of samples.

Two types of water were used for irrigation water, namely ground water pumped up from boreholes and collected rainfall water stored in ponds (Table 7). Ground water contained no STEC and exactly 1 cfu *E. coli* per 100 ml in 4 out of 22 samples, while STEC was detected in 19.6 % (11/56) of the rain water samples and the majority (40/56) was contaminated with on average 1.6 log cfu/100 ml *E. coli*. Prevalence of STEC (Chi^2, Likelihood ratio, $p = 0.118$) and counts of *E. coli* (Kendall's tau-c, $p = 0.080$) were not significantly different between water samples taken from the source and water versus at the actual (starting) point of the drip irrigation. Four types of reservoirs for the storage of the irrigation water were observed: i) a pond without plastic coverage on the bottom (n = 24), ii) a pond with plastic coverage on the bottom (n = 16), iii) a raised (having elevated ridges as borders) pond with plastic coverage on the bottom (n = 16) and iv) a borehole (so no real storage of irrigation water) (n = 22) (Figure 18). Generic *E. coli* counts (Kendall's tau-c, $p < 0.001$) and the PCR detection of STEC (Chi^2, Likelihood ratio, $p = 0.020$) were significant higher in the various ponds than in the borehole. However, *E. coli* counts and STEC prevalence were lower in the raised pond in

comparison to other ponds, suggesting that the elevated borders offered protection against microbial contamination.

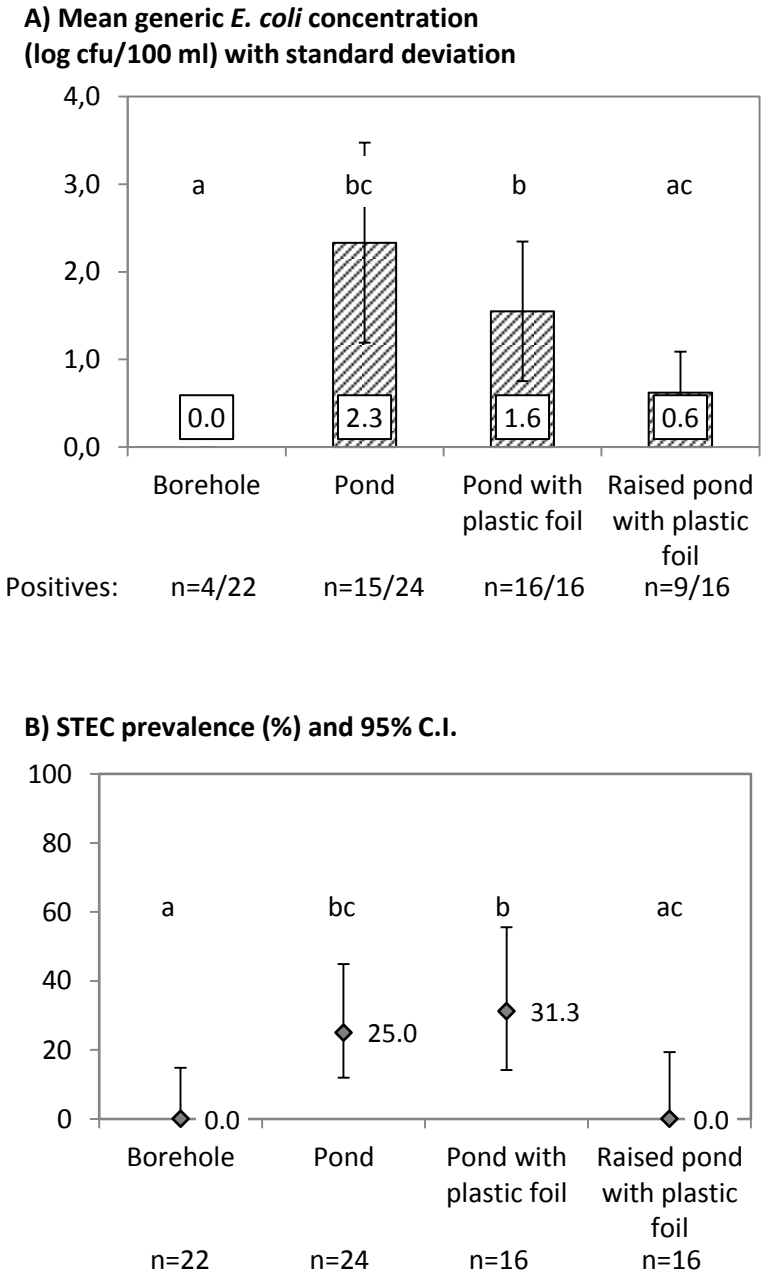

Figure 18: A) Mean generic *E. coli* concentrations (log cfu/100 ml) with error bars indicating the standard deviations in irrigation water samples which were positive for *E. coli* stored in various reservoirs. B) STEC prevalence (%) in ground and rain water with error bars indicating the 95 % C.I. on the prevalence estimate. Different letters indicate statistically significant differences and n = the number of samples (2012).

Irrigation water was treated on farm 4 and 5 by sand filtration (Table 7). Water treatment was significantly associated with a lower number of *E. coli*. Irrigation water from farms

without treatment contained on average 1.77 log cfu/100 ml *E. coli* ± standard deviation 1.20 log cfu/100 ml, while farms with treated irrigation water showed on average 0.89 ± 0.71 log cfu/100 ml *E. coli*. STEC was also significantly more prevalent in untreated water (10/48 = 20.8 %; 95 % C.I.: 11.7 – 34.3 %) than in treated water (1/30 = 3.3 %; 95 % C.I.: 0.6 – 16.7 %). The distance between the strawberry fields and the nearest toilet, the average daily temperature, daily precipitation and the temperature of the irrigation water were not of significant influence on STEC prevalence nor on the *E. coli* counts. Minor flooding of the strawberry field had occurred once in July 2012 when sampling at farm 3, but this event did not have significant effects on the microbiological contamination as beds of strawberries' plants were raised and thus there was no contact of water with the plants and strawberries.

Multivariable analysis was performed to investigate which significant risk factors (Table 11) from the univariable analysis remain when all factors were considered together, to estimate their quantitative effects and check whether there are interactions between the risk factors. The risk of STEC prevalence was determined by the generic *E. coli* count (odds ratio (OR) for 1 log increase = 4.64) and the season (highest in summer, followed by spring (OR = 0.13) and fall (OR = 0.05)) (Figure 19). Seasons were defined as follows: spring includes the months April, May and June, summer comprises July, August and September and fall is October, November and December.

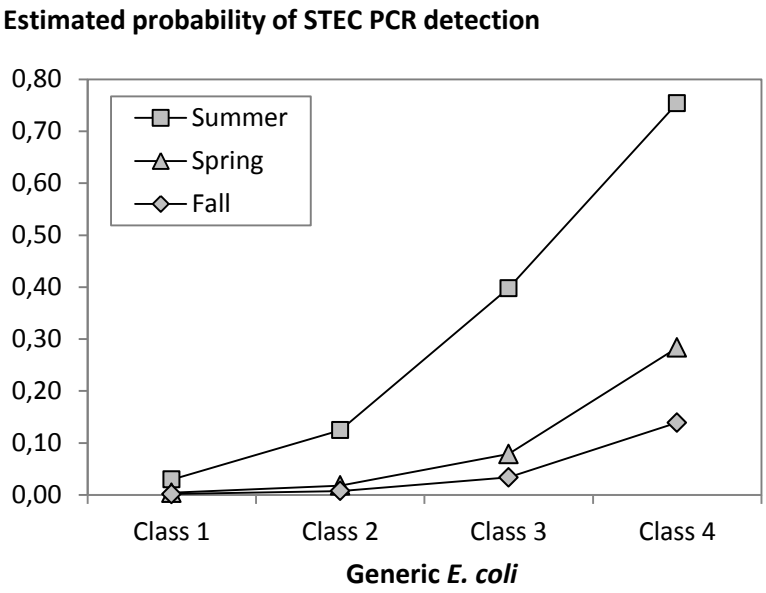

Figure 19: Estimated probability of STEC presence by multivariable logistic regression in function of the risk factors, i.e. season and generic *E. coli* concentration (2012).

The presence of generic *E. coli* was determined by the sample type, the season, the irrigation water type and application of water treatment. *E. coli* was most frequently found in water, then in soil/substrate (OR = 0.10), and was rarest on hands (OR = 0.01) and strawberries (OR = 0.004) (Figure 20). Generic *E. coli* was most prevalent in the summer and fall seasons and lower in spring (OR = 0.05). *E. coli* was more frequently present in collected rain water stored in ponds than in ground water pumped from boreholes (OR = 5.86) and if irrigation water was not treated by sand filtration (OR = 19.24).

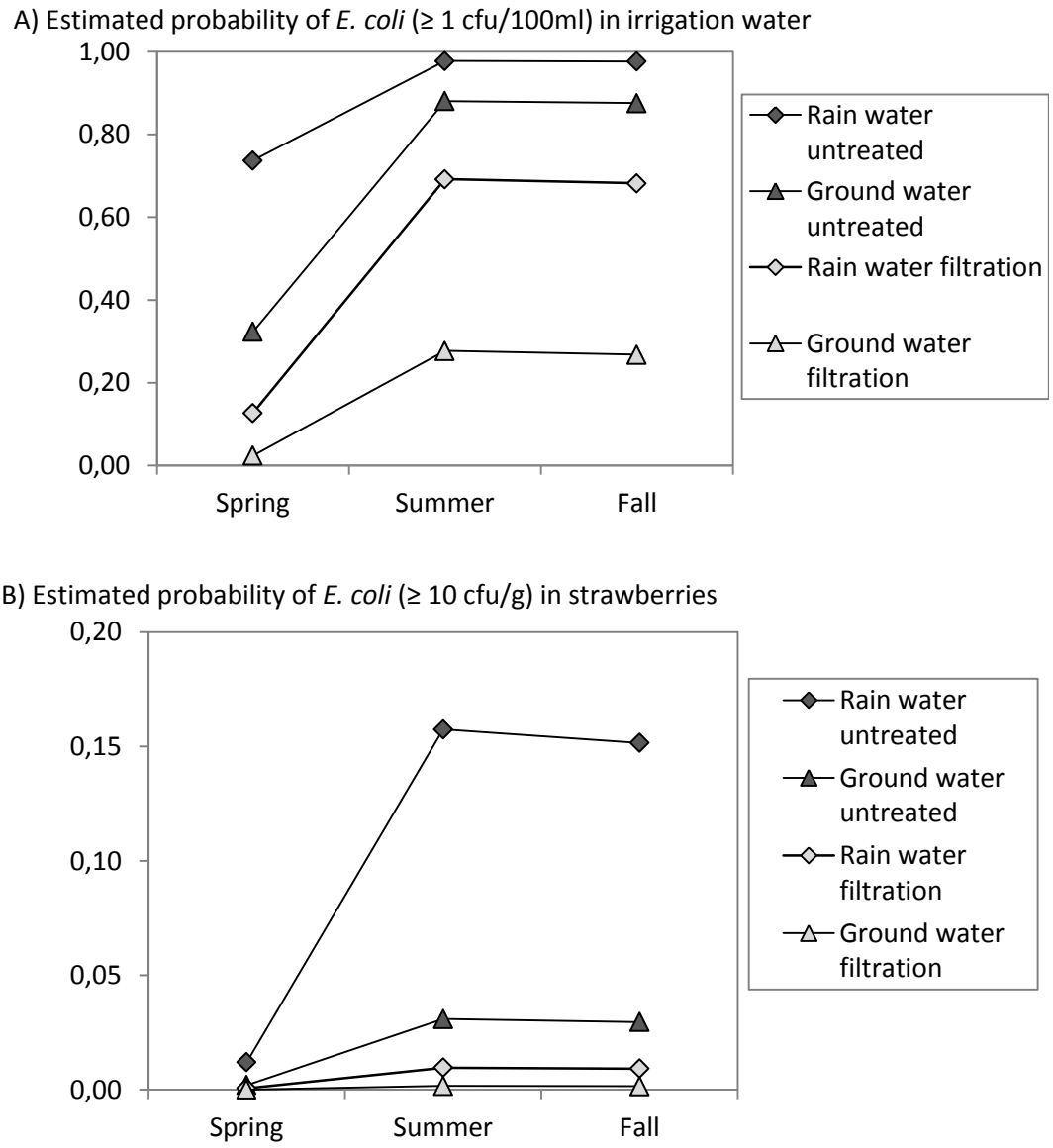

Figure 20: Estimated probability of generic *E. coli* presence by multivariable logistic regression in function of the risk factors, i.e. season, water type (ground or rain water), water treatment (untreated or sand filtration) and sample type, showing the presence in irrigation water (≥ 1 cfu/100 ml), the sample type with the highest risk (A) and in strawberries (≥ 10 cfu/g), the sample type with the lowest risk (B) (2012).

4.3.3 Follow up study: source of STEC contamination in the irrigation water on farm 1

Further research on the STEC O26 isolates from the irrigation water and substrate of farm 1 was performed in 2013, as it was also established to have multiple risk factors: a mixed farm with cattle, using collected rainfall water in open reservoir without raised edges and no water treatment. As in 2012, no *Salmonella* was found in the samples taken throughout the 2013 fruiting season at this production site (0/48 irrigation water, 0/8 geese pond water, 0/24 substrate, 0/16 geese feces, 0/24 beef cattle feces). STEC was detected by PCR in 7/48 irrigation water samples, none (0/8) of the geese pond water samples, 1/24 substrate samples, 1/16 geese feces and in all (24/24) beef cattle feces. Similar to the previous sampling, *stx* genes were always accompanied with *eae* genes. *AggR* genes were never detected in any sample. *Eae* genes without *stx* genes were detected in 36/48 irrigation water samples, 16/24 substrate samples, 12/16 geese feces and 7/8 pond water samples. From none of the STEC PCR-positive samples, isolates were obtained (0/33), but in 29/33 (6/29 irrigation water, 23/29 cattle feces) the corresponding serotype and *eae* variant combination was found (Table 10). Similar STEC serotypes were detected by PCR in pens of young (< 10 months) and older (≥ 10 months) cattle. The main serotype which was detected in combination with the corresponding *eae* variant was O103 (20/24), followed by O145 (10/24), O26 (7/24) and O111 (1/24). In addition, variation in serotypes (detected by PCR) was observed: during the first three visits (May and beginning of June) O26 was mainly detected (as well in the younger as in the older cattle), whereas O103 was mainly detected during the last nine visits (end of June to October). *E. coli* was enumerated in all water samples (48/48 irrigation water (1.5 ± 0.8 log cfu/100 ml), 10/10 pond water of the geese (3.5 ± 0.5 log cfu/100 ml)) and in 14/22 substrate samples (1.7 ± 0.7 log cfu/g). *E. coli* exceeded a value of respectively 100 and of 1000 cfu/100 ml in 12/48 and 2/48 samples of the collected rainfall water to be used as irrigation water. The level of generic *E. coli* was much higher in the bathing pond of geese than in the irrigation water pond, all (10/10) samples contained more than 100 cfu/100ml and 9 out of 10 contained more than 1000 cfu/100ml. However, despite the elevated counts of *E. coli* in the geese pond water, no STEC were detected.

4.4 Discussion

The sanitary quality of Belgian strawberries was good, since no *Salmonella* nor STEC was detected. *Salmonella* was never detected in any of the samples for any of the sample types, neither throughout the 2012 initial screening survey or in the focused 2013 survey in farm 1 (0 out of 318 samples). STEC was not detected on strawberries (60 g), whereas generic *E. coli* was detected in two strawberry samples (one with 1 and one with 1000 cfu/g) from farm 3. There are no Hygiene Criteria for fresh strawberries, but a Process Hygiene Criterium for pre-cut ready-to-eat (RTE) fruit and vegetables exist with m = 100 cfu/g, M = 1000 cfu/g, n = 5 and c = 2 (EC/2073/2005). The two samples positive for *E. coli* came from farm 3, which had no risk factors except the use of untreated water (pond or ground water) for irrigation and never samples positive for STEC (0/28). In contrast to the strawberry samples, STEC and generic *E. coli* were regularly present in the production environment, i.e. in irrigation water and substrate. The cultivation type (soil vs. soilless culture) did not impact on the occurrence of STEC or generic *E. coli*. Collected rainfall water in ponds which can receive surface run-off water (no elevated borders) and ponds which are located near cattle grazing or cattle residence poses the highest risk. The irrigation water was frequently contaminated with STEC (11 out of 78 samples in the 2012 survey) and *E. coli* (44 out of 78 samples in the 2012 survey), but all producers applied drip irrigation to the strawberry plants, so the contamination risk was strongly reduced, because no direct contact between irrigation water and the strawberries occurred. Internalization studies for *Salmonella* or STEC via roots of plants and investigation of potential transfer in this way to strawberries have not been performed yet. Internalization of pathogens via the irrigation water or the soil through the roots into the edible leaves and fruits of plants is generally unlikely, it occurs sporadically and at low levels (Hirneisen et al., 2012). However, it should be noted that (1) internalization is plant and pathogen specific; (2) the cultivation type has an impact (soil versus soilless); (3) internalization is less likely for intact and healthy roots; and (4) the presence of internalized pathogens in roots of plants does not directly correlate with internalized pathogens in the edible or foliar tissues. For example, irrigation with water containing as much as 10 log cfu/100 ml *Salmonella* did not result in contamination of the fruit, stems and leaves, but only sporadically in the contamination of the roots (Miles et al., 2009), so internalization in the fruit by irrigation of the plants with naturally contaminated water with low numbers of

pathogens can be considered as highly unlikely. It is more likely that contaminated substrate or soil contaminates the strawberries by splashing of soil particles onto the strawberries during rainfall. However, most strawberries are grown in protected culture, either in greenhouses or in plastic tunnels in raised beds to facilitate picking. Moreover, even for strawberries grown in soil and in unprotected culture (in the open field without plastic tunnels) the opportunity for soil contact due to rainfall is limited, because the soil in which strawberries are grown is covered by plastic foil. As expected from these limited opportunities for contamination, the strawberries showed high microbial safety and sanitary quality.

Increased *E. coli* counts and the summer months were the main risk factors for PCR based STEC detection in the primary production of Belgian strawberries. Comparable results were found in Belgian lettuce farms (Holvoet et al., 2014). Also in the latter study elevated *E. coli* levels increased the probability for the presence of pathogens *Campylobacter*, STEC and *Salmonella*. Similarly, also in sampling irrigation water used in lettuce primary production the lowest levels of *E. coli* in the irrigation water were found in March, April and December, the coldest months in the beginning and at the end of the growing season in Belgium (Holvoet et al., 2014). The current EU legal framework does not include microbiological criteria applicable at the primary production stage (Hygiene Criterion). However, using *E. coli* as an indicator of recent human or animal fecal contamination is likely to be useful for verification of good agricultural practices (GAP) and good hygienic practices (GHP) applied to berries at individual production sites, for example to assess the suitability of water used for irrigation (EFSA, 2014b). Thus monitoring of *E. coli* as indicator organism is appropriate, but it should be done frequently and with consideration of the irrigation water type and the risk factors present on the farm.

Microbial criteria have only been established in Spain for the use of treated wastewater for the irrigation of crops that are likely to be eaten uncooked, namely *E. coli* should be ≤ 100 cfu/100 ml (Spanish Official Bulletin, 2007). These criteria seem too high to limit the risk of STEC contamination via the irrigation water used by Belgian strawberry producers, because 23% (3/13) of the samples containing between 10 and 100 cfu/100 ml generic *E. coli* were positive for STEC, while no STEC was found in samples with < 10 cfu/100 ml *E. coli* (0/49) Figure 14). If such criteria are established, they should be set after assessing the local

relationship of *E. coli* with the pathogen(s) of interest. In the US, water that may come in contact with the harvestable portion of produce must meet a standard of no more than 235 cfu of generic *E. coli* per 100 ml throughout the growing season (FSMA, 2013). Furthermore, the FSMA requires frequent testing depending on the risk level of the irrigation water source: at least weekly for surface water and for ground water at least monthly when stored in an underground reservoir and at least every three months for groundwater freshly pumped up. The risk factors which were identified to indicate a higher likelihood of contamination with pathogens should be taken up in sanitary surveys, training, observational audits and other methods to verify agricultural and hygiene practices for berries at primary production. Water treatment prior to irrigation should be regarded as an option, even though drip irrigation and protected culture is done. In the present survey, the pathogens *Salmonella* and STEC were not detected on strawberries and the fecal indicator *E. coli* was only sporadically present, but vigilance and preventive measures are still recommended.

In the present study, STEC O26 was isolated by culture from the 2 out 4 water and the 2 out of 2 substrate at one sampling time on farm 1, initiating a follow-up study in next fruiting season. On this farm also a number of risk factors were observed: keeping of beef cattle next to the strawberry fields and approximately 50 m from the irrigation water pond and using untreated rainfall water collected in a non-elevated pond which bordered to geese and their bathing pond from which run-off was observed after rainfall. In the follow-up study, STEC was detected by PCR in all samples of cattle feces (24/24), but in only one sample of geese feces (1/16) and not in the bathing pond of the geese (0/8). It was noted that the STEC of the only positive geese feces sample did not belong to serotypes O26, O103, O111 or O145, while the former STEC serotypes were detected in the cattle fecal samples and in the irrigation water (Table 10). This indicates that the cattle present on the farm, rather than the geese living near the irrigation water source, were the likely reservoir of STEC on the farm and source of contamination of the irrigation water on strawberry farm 1. This study confirms the current opinion that cattle is the main reservoir for STEC (Chapman et al., 1993; Cobbaut et al., 2008). In France, *stx* genes were detected in most but not all fecal samples of cattle (330/471) (Pradel et al., 2000). In the present study, *stx1* and *stx2* genes were detected by PCR in all fecal samples of the cattle (24/24), but it should be noted that fecal

samples of the cattle were taken by the overshoe method, which is more efficient than analyzing individual rectal samples as was the case in the other studies (Cobbaut et al., 2008).

A variety and sometimes simultaneous presence of multiple serotypes were detected in this study and these serotypes changed in the course of sampling weeks. The O145 serogroup was always detected by PCR (although not always in combination with its *eae* variant), while O26 occurred in the beginning of the sampling period (May - June) and O103 at the middle and end (June - October). STEC O103 has been isolated the most in rectal samples of Belgian beef cattle (6/12) (Joris et al., 2011a). In the present study, *E. coli* O157 was not isolated from any of the cattle samples, just as in the study of Cobbaut et al. (2008). During monitoring individual animals intermittent excretion of STEC has been observed (Joris et al., 2011a; Pradel et al., 2000; Robinson et al., 2004). But as in the present survey, variable serotypes was observed by Joris et al. (2011a), during sampling of the same cow from STEC O26 to STEC O111. The European Commission (EC, 1997) reported that *E. coli* O157 has been isolated from geese, in contrast to Wahlstrom et al. (2003) where no *E. coli* O157 was isolated from Canada geese fecal samples (0/105) in Sweden. Also in ducks, *stx* and *eae* genes were detected by PCR in 2/20 rectal samples, whereupon *E. coli* O157 has been isolated from one of these fresh fecal matters (Samadpour et al., 2002). Wild birds, rodents, cats or dogs may become infected by cattle and possibly be part of the STEC transmission and dissemination (Joris et al., 2013; Nielsen et al., 2004).

In the present survey, STEC was always accompanied by *eae* genes and never by *aggR*. In fact, the *aggR* gene was never detected, so it is rare in the environment, in contrast to the *eae* gene, which was frequently detected. These findings support the current assumption that STEC with *aggR* has in particular a human reservoir with a low likelihood of occurrence in cattle and in the primary production environment (EFSA, 2013c). The *eae* gene was detected in the absence of *stx* genes in 106 samples (2 strawberry, 58 irrigation water, 21 substrate, 6 plastic foil cover on soil, 12 geese feces and 7 pond water samples) by PCR analysis of the enrichment broth. The reason may be the presence of adhesive Enteropathogenic *E. coli* (EPEC) or other bacteria such as *Citrobacter rodentium* which can also possesses the *eae* gene (Beutin et al., 2009). The consequences of positive results from STEC PCR screening may be overestimated if *stx* and *eae* genes originated from multiple

strains of *E. coli*. Therefore, in this study, samples positive for *stx* and *eae* were further analyzed by determining the *eae* variant (β, ε, θ and ϒ) and the serotype (O26, O103, O145 and O111) using PCR. Samples with the appropriate *eae* and serotype combination were regarded as more likely to be truly positive for pathogenic STEC (EFSA, 2013c). STEC belonging to serotypes O26, O103, O111 and/or O145 with the corresponding *eae* variant were detected by PCR in 14 irrigation water samples, 2 substrate samples and 23 cattle feces samples, but only 4 isolates (STEC O26) were obtained by culture. Even the 4 PCR positive water samples taken from at one day at one producer only provided 2 isolates. This may indicate a failure in the performance of culture methods for non-O157 STEC, e.g. overgrowth of the target bacteria by background microbiota during enrichment or on the selective agar media or the loss of *stx* during confirmation steps. The STEC PCR results often showed high Ct values and it is known to be quite challenging to provide isolates from low contaminated samples, in particular when high numbers of generic *E. coli* are present (Joris et al., 2013). As Pearce et al. (2004) reported that *E. coli* O157 was mostly excreted in low amounts (< 100/g), isolation methods need to be sensitive enough to isolate pathogenic *E. coli* in these low amounts from the background microbiota with high numbers of commensal *E. coli* (approximately 10^5 – 10^6 cfu/g cattle feces) (Sinton et al., 2007; Wang et al., 2004). Moreover, Joris et al. (2011b) reported the loss of *stx* genes of STEC strains during the first subcultivation step of natural contaminated fecal bovine samples, whereby it was noticed that *stx* genes appeared to be more stable in O157 strains than in non-O157 strains.

ANNEX 1

AARDBEIEN

VRAGENLIJST VOOR DE LANDBOUWER

Variëteit:

Productieperiode: van tot

TEELT

1. IRRIGATIEWATER

1.1 Geschatte hoeveelheid water gebruikt voor irrigatie: m³/jaar

1.2 Wat is de bron van irrigatiewater? (Meerdere antwoorden mogelijk)

- ☐ Grondwater
- ☐ Oppervlaktewater:
  - ☐ Beekwater ☐ Open putwater ☐ Rivierwater
- ☐ Boorputwater (regenwater)
- ☐ Stadswater
- ☐ Industriëel afvalwater
- ☐ Andere, specifieer:

Beantwoord **vraag 1.2.1, 1.2.2 en 1.2.3** ingeval van gebruik van **afvalwater!**

1.2.1 Indien gebruik van afvalwater, werd deze behandeld?

☐ Ja, specifieer:

☐ Nee

1.2.2 Wordt gebruik gemaakt van opslagplaatsen ontworpen voor het beheersen, reduceren of elimineren van microbiële pathogenen?

☐ Nee ☐ Ja, specifieer:

1.2.3. Voldoet het afvalwater aan specifieke criteria?

(bv. microbiologische criteria voor behandeld afvalwater: (WHO) maximum 1000 fecale coliformen/100 ml en maximum 0,1 intestinaal nematode ei per liter; (Wetenschappelijk Comitée) maximum 10 kve E. coli/ml)

☐ Nee ☐ Ja, specifieer:

1.3 Hoe wordt het irrigatiewater gestockeerd? (open - gesloten put, diepte, …)

1.4 Wordt de microbiologische kwaliteit van het irrigatiewater bepaald door analyse?

☐ Ja ☐ Nee

Beantwoord vraag **1.4.1-1.4.2** indien **microbiologische analyses** worden uitgevoerd!

1.4.1 Wordt de waterbron getest voor:

☐ Totale coliformen ☐ *Clostridium perfringens* ☐ *E. coli*

☐ Fecale coliformen ☐ *Salmonella* spp. ☐ *E. coli* O157

☐ intestinale enterococci ☐ intestinale nematoden

☐ Andere (enkel indien totale en fecale coliformen > 1000/ml en > 100/ml respectievelijk), specifieer:

1.4.2 Hoeveel keer worden microbiologische analyses uitgevoerd? (Analyserapport aanwezig?)

☐ Jaarlijks ☐ 2 maal per jaar (1 maal per semester)

☐ Maandelijks ☐ Wekelijks

☐ Ander (leg uit):

1.5 Wordt de aanwezigheid van dieren (bv. vogels, huisdieren, …) vermeden rond de waterbron?

☐ Ja ☐ Nee

1.6 Wordt de vegetatie rond de waterbron onder controle gehouden?

☐ Ja ☐ Nee

1.7 Wordt run-off van velden of de veestapel naar de waterbron verhindert?

☐ Nee ☐ Ja, specifeer:

1.8 Welke behandelingen worden toegepast voor de waterbron (leg uit)?

	Waterbron (1):	Waterbron (2):
Niets		
Filtratie		
Chemische bestrijding: Chemisch agent: Concentratie: Frequentie:		
Filtratie + UV-licht		
Andere: pH shock, biologische controle, aëratie, coagulatie, flocculatie, …		

1.9 Welk irrigatiesysteem wordt gebruikt?

☐ Sproei-irrigatie (beregening)

☐ Druppelbevloeiing

☐ Hydrocultuur

☐ Vorenirrigatie (via goten)

☐ Sprinkler irrigatie

☐ Andere, specifieer :

1.10 Wanneer is de laatste irrigatie?

☐ Zelfde dag van oogsten

☐ 1 dag voor oogsten

☐ 2 à 4 dagen voor oogsten

☐ 5 à 7 dagen voor oogsten

☐ Méér dan 7 dagen voor oogsten

2. BEMESTING

2.1 Welk type mest wordt gebruikt?

Soort	**Ja/nee**	**Concentratie & Frequentie**	**Tijdstip**
Minerale meststoffen			
Organische meststoffen			
Slib			
Compost			
Stalmest			
Kippenmest			
Gemengde mest (specifieer)			
Andere (specifieer):			

2.2 Hoeveel tijd bevindt zich tussen het bemesten en planten van de aardbeien?

☐ dagen

2.3 Wat is de oorsprong van de aardbeienplanten?

Specifieer (zelf opgekweekt, aangekocht, waar aangekocht,…):

3. TEELT TYPE

3.1 Welk type teelt wordt gebruikt?

☐ Volle grond ☐ Hydrocultuur

3.2 Indien hydrocultuur, welk substraat wordt gebruikt?

3.3 Wordt naast de aardbeienteelt eveneens aan veeteelt gedaan?

☐ Nee ☐ Ja, specifieer:

3.4 Indien u veeteelt beoefent, welke voorzorgsmaatregelen worden getroffen bij het betreden van de aardbeienteelt?

☐ Handen wassen ☐ Verse kledij

☐ Laarzen ontsmetten ☐ Andere schoenen/laarzen

☐ Andere (specifieer):

OOGST

1. Wat is de productiehoeveelheid per jaar?

2. Hoeveel personen helpen bij de manuele oogst?

3. Worden handschoenen gedragen tijdens het plukken van de aardbeien?

☐ Ja ☐ Nee ☐ Sommige (%):

4. Worden andere voorzorgsmaatregelen genomen bij het oogsten?

☐ Handen wassen ☐ Beschermkledij dragen

☐ Andere (specifieer):

5. Hoelang duurt 1 werkshift (in uren)?

6. Hoever is het toilet? (in meter afstand van het veld)

7. Worden de aardbeien onmiddellijk gekoeld na het plukken?

☐ Nee ☐ Ja, specifieer:

Beantwoord **7.1 en 7.2** indien onmiddellijk **gekoeld** wordt!

7.1 Welk koelingssysteem wordt gebruikt?

7.2 Wat is de tijd tot koelen (in minuten)?

8. Welk verpakkingsmateriaal wordt gebruikt?

9. Schematisch overzicht van teeltsysteem:

CHAPTER 5: Microbiological analysis of pre-packed sweet basil (*Ocimum basilicum*) and coriander (*Coriandrum sativum*) leaves for the presence of *Salmonella* and Shiga toxin-producing *E. coli*

Redrafted from:

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ABSTRACT

Enteric pathogens, such as *Salmonella* and pathogenic *E. coli*, have been detected and associated with foodborne outbreaks from (imported) fresh leafy herbs. Screening on imported herbs from South-East Asian countries has been described. However, limited information on prevalence of these pathogens is available from other sourcing regions. Therefore, fresh pre-packed basil and coriander leaves from a Belgian trading company were investigated for the presence of *Salmonella,* Shiga toxin-producing *E. coli* (STEC), generic *E. coli* and coliforms. In total 592 samples were collected originating from Belgium, Israel and Cyprus during 2013-2014. Multiplex PCR followed by further culture confirmation was used for the detection of *Salmonella* and STEC, whereas the Petrifilm Select *E. coli* and VRBL-agar were used, respectively, for the enumeration of *E. coli* and coliforms. *Salmonella* was detected in 10 out of 592 samples (5 basil and 5 coriander), of which two samples were sourced from Israel and eight from Cyprus. The presence of STEC was suspected in 11 out of 592 samples (3 basil and 8 coriander), due to the detection of *stx* and *eae* genes, of which one sample originated from Belgium, four from Israel and six from Cyprus. No STEC were isolated by culture techniques, but in three samples a serotype (O26, O103 or O111) with its most likely associated *eae*-variant (β or θ) was detected by PCR. Generic *E. coli* was enumerated in 108 out of 592 samples, whereby 55, 32 and 13 samples respectively between 1 - 2, 2 - 3 and 3 - 4 log cfu/g and 8 samples exceeding 4 log cfu/g. Coliforms were enumerated in all herb samples at variable levels ranging from 1.6 to 7.5 log cfu/g. Further statistics indicate that *E. coli* was significant correlated with the presence of *Salmonella* ($p < 0.001$) or STEC ($p = 0.019$), while coliforms counts were significant correlated with *Salmonella* ($p < 0.001$), but not with STEC ($p = 0.405$). Generic *E. coli* is a better indicator for the presence of enteric pathogens than coliforms on fresh herbs, but the relationship between *E. coli* and *Salmonella* or STEC was not strong enough to provide a threshold value for *E. coli* to assure food safety (i.e. no pathogens present). Results indicate that fresh leafy herbs like basil and coriander sourced from different cultivation regions, may contain enteric pathogens and potentially pose a risk for human health.

5.1 Introduction

The objective of the present study was to have an idea of the prevalence of pathogens and microbiological quality of pre-packed fresh herbs leaves as a ready-to-use convenience product being marketed in Belgium. As *Salmonella* and pathogenic *E. coli* (including *E. coli* O157:H7) were found to be among the major cause of foodborne outbreaks associated with fresh produce in the USA and EU (Olaimat & Holley, 2012) and consumption of fresh herbs such as coriander and basil gains popularity, the present study investigated the prevalence of *Salmonella,* Shiga-toxin producing *E. coli* (STEC) (with the main focus on serotype O157, O26, O103, O111, O145), generic *E. coli* and coliforms on pre-packed basil and coriander leaves both from Belgium (in production season June to September) and sourced from Israel and Cyprus (year around). Furthermore, the relation between the presence of enteric pathogens on basil and/or coriander and generic *E. coli* or coliforms as indicator organisms was investigated.

5.2 Material and methods

5.2.1 Sampling

In 2013, pre-packed fresh basil and coriander leaves were sampled at the most important Belgian trading company putting this type of product to the Belgian retail market. Basil and coriander are also sold on the Belgian market as intact plants of herbs in pots, but these were not included in the present study. The focus of the present study was on pre-packed fresh herb leaves as a ready-to-use convenience product. The at the stem cut herb leaves arrive in bulk packages from the sourcing areas (Cyprus and Israel by air flight or from Belgium by truck) in the trading company, stored at maximum 7°C. They are further manually packed in small plastic trays within 24 h after arrival in the trading company under hygienic conditions. Pre-packed fresh herb leaves are sent off to retail stores within a maximum of 24 h after packing (intermediate storage at maximum 7°C and refrigerated transport to retail taking max. 2 h). Batches of basil and coriander leaves were sampled at the trading company at the time of packaging. The two major (almost) year-around sourcing regions for basil and coriander for this trading company were included in the survey, namely

Israel and Cyprus. In addition in the summer, when there is some basil and coriander grown and available from Belgium, we also sampled these, aiming at getting the same number of samples from Belgium (although the growing season was much shorter) as for Israel/Cyprus in order to have similar (un)certainty on the estimates of the prevalence of pathogens and overall microbiological quality, established by enumeration of indicator organisms, for the three sourcing regions. The sampling was performed each week (if possible) and spread over the time period that the commodity was available (Figure 21). For both basil and coriander, in total 300 samples of fresh-cut leaves were delivered to our lab on a weekly basis as freshly packed fresh-cut leaves the same day as they were packed. Samples were transported to the lab on ice in a cool box, stored at 4°C and 12°C for respectively coriander and basil (basil being cold-sensitive) and subjected to microbial analysis within 5 h of receipt. The 300 samples taken for each of the herbs under investigation consisted of 100 samples for each country of origin. Thus, approximately two samples of pre-packed basil and coriander leaves imported from Israel were analyzed during 51 weeks (February 2013 to January 2014), whereas in case of herbs originating from Belgium - due to the restricted period of favorable climatic conditions and thus the shorter growing season - 5 to 8 samples were analyzed on a weekly basis and this for each type of herb during 14 weeks (June 2013 to September 2013).

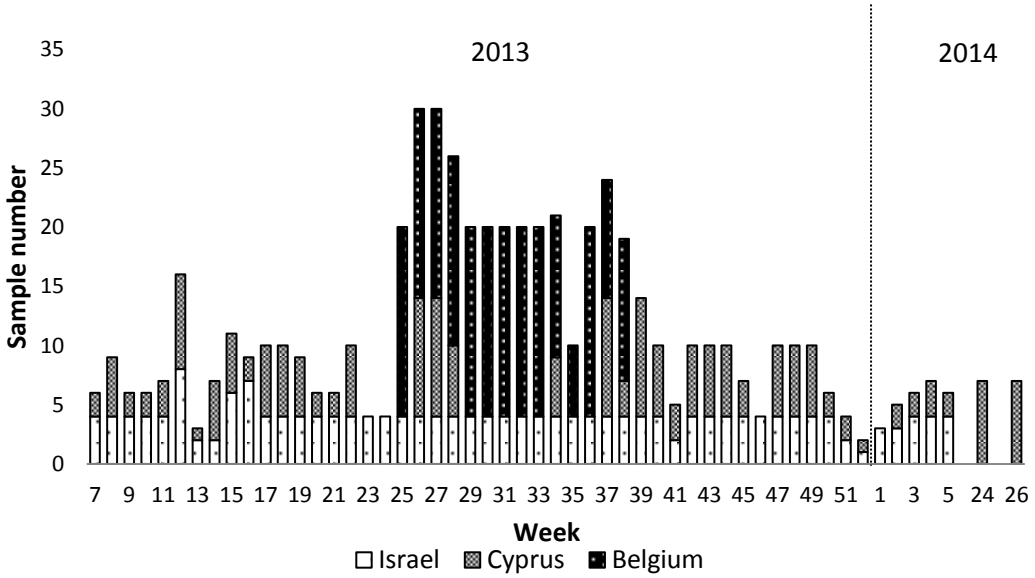

Figure 21: Overview of samples (basil and coriander) taken per week and per country (Israel, Cyprus and Belgium) during a whole year

For the sourced herbs from Cyprus also a year around sampling was intended, as was executed for Israel, but due to too hot and cold climatic conditions in May 2013 (week 19-

20) and in December 2013 (week 49-50), respectively, cultivation and import was interrupted (Figure 21). Moreover, logistic and quality problems led to missing samples of coriander and/or basil in several weeks of 2013 (Figure 21). To account for the missing samples in those weeks, more samples were taken and spread during the following weeks (i.e. instead of 2 samples per week 3 or more were taken). In case of basil originating from Cyprus it was not possible to collected 100 samples in one year, therefore the sampling was proceeded in June 2014.

5.2.2 Microbiological analysis

Coliforms and the fecal hygiene indicator *E. coli* were enumerated on basil and coriander. Samples (25 g) were diluted in 225 ml Buffered Peptone Water (BPW; Oxoid, UK) (10^{-1}) in a filter stomacher bag followed by one minute stomaching. The Petrifilm Select *E. coli* (LED techno, Belgium) was used for the enumeration of *E. coli* by applying 1 ml of the tenfold dilution of the product followed by incubation at 37°C for 24 h ± 2 h. Coliforms were enumerated by applying 1 ml of the appropriate tenfold dilution of the product (10^{-1} to 10^{-4}) on pour plates of Violet Red Bile Lactose (VRBL; Oxoid) agar with an overlayer followed by incubation at 37°C for 24 h ± 2 h. Tenfold dilutions 10^{-2} to 10^{-4} were made in Peptone Physiological Salt solution (PPS; 8.5 g/l NaCl (Fluka, Switzerland) and 1 g/l neutralized bacteriological peptone (Oxoid)).

Basil and coriander were analyzed for the presence of *Salmonella* and Shiga toxin-producing *E. coli* (STEC) per 25 g. Samples were enriched in a filter stomacher bag (FBAG-04, Novolab, Belgium) tenfold diluted with BPW at 37°C for 18 - 24 h. *Salmonella* and STEC were detected by multiplex real-time PCR of the *iroB* gene for *Salmonella* and the *stx1*, *stx2*, *eae* and *aggR* genes for STEC with the GeneDisc plate *Salmonella* & aggregative *E. coli* as described in Beutin et al. (2009). In short, fifty microliter of the enriched sample was put in a lysis tube (Extraction Pack Food 1, Pall Technology) for the DNA extraction by heating (10 min, 100°C) followed by centrifugation (2 min, 10,000 *x g*). Subsequently, the supernatants was twofold diluted, using the dilution buffer (Extraction Pack Food 1) to prevent inhibition of the PCR reaction, as observed in a preliminary study. *Salmonella* PCR presumptive positive signals were subjected to culture confirmation as described in ISO 6579:2002. Briefly, non-selective enrichment in 10 ml BPW at 37°C for 18 – 24 h was followed by a selective enrichment in 10

ml Rappaport-Vassiliadis Soya Peptone Broth (RVS; Biomérieux, Belgium) at 42°C and in 10 ml Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn; Biomérieux) at 37°C. After 24 ± 3 h incubation, samples were plated on Xylose Lysine Deoxycholate agar (XLD; Oxoid) and *Salmonella Brilliance* (Oxoid) and presumptive *Salmonella* colonies were confirmed by Crystal ID E/NF (BD, Belgium). Confirmed *Salmonella* isolates were further transferred to a reference lab (WIV-ISP (Belgium) or IDS, RIVM (the Netherlands)) for serotyping. STEC is defined by the Shiga toxin genes *stx1* and/or *stx2* and in our study the combination of *stx1*/*2* with adhesion genes *eae* or *aggR* was determined due to the enhanced pathogenic potential of adhesive STEC (EFSA, 2013c; Tozzi et al., 2003). Moreover, PCR positive STEC samples were further analyzed for the presence of serotypes O26, O103, O111 and O145 and for the *eae*-variants (β, σ, ϒ, ε) by PCR (the GeneDisc STEC identification plate and the GeneDisc STEC plus plate (Pall Technologies), respectively), because these serotypes have increased pathogenic potential (EFSA, 2013c; Posse et al., 2007). Specific serotypes are associated with specific *eae* gene variants (*eae*-β with O26, *eae*-θ and *eae*-ε with O103, *eae*-γ with O157 and O145 and *eae*-θ, *eae*-γ and *eae*-ε with O111) (Bibbal et al., 2014; Madic et al., 2010; Oswald et al., 2000; Posse et al., 2007). In this way, the (im)possibility of the *stx* and *eae* virulence factors originating from one *E. coli* strain is gauged by the match of the *eae* variant and the serotype detected by PCR. Furthermore, after PCR detection of STEC (*stx* and *eae* detection), culture confirmation was performed for STEC O157 and non-O157 as follows. Briefly, immunomagnetic separation (IMS) with Dynabeads anti-*E. coli* O157 (Invitrogen Dynal AS, Norway) was followed by plating on Cefixime Tellurite Sorbitol MacConkey agar (CT-SMAC; Oxoid) and on SMAC with chromogen 5-bromo-4-chloro-3-indolyl-b-D-glucuronide *(*BCIG; Oxoid). In parallel for non-O157 *E. coli*, samples were streaked on CHROMagar STEC (CHROMagar Microbiology, France) and/or CHROM ID STEC (Biomérieux). In addition, both the enriched sample as isolated *E. coli* presumptive colonies were further sent off for potential STEC isolations or confirmation of STEC serotypes by the national expert lab on STEC (the Lab of Hygiene and Technology, Faculty of Veterinary Medicine, Ghent University). In this lab, samples were plated on CT- SMAC for STEC O157 and on MAC for non-O157 STEC (as well the obtained presumptive isolates as the enriched sample). STEC presumptive colonies obtained from CT-SMAC and/or MAC were further analyzed by multiplex PCR (*stx1*, *stx2* and *eae*). If virulence genes were detected, isolates were analyzed for the serotype by PCR.

5.2.3 Statistical analysis

The 95 % confidence interval (C.I.) on prevalence estimates was calculated by the Wilson score method without continuity correction (Wilson, 1927). Results were processed with SPSS 22 at a significance level of 5 % (p = 0.050). Ordinal classes (1-4), starting from the detection limit and comprising tenfold differences in concentrations (< 1.0 log cfu/g (= undetected), ≥ 1.0 and < 2.0 log cfu/g, ≥ 2.0 and < 3.0 log cfu/g and ≥ 3.0 log cfu/g, respectively) were defined for the enumeration data for generic *E. coli*, because *E. coli* was undetected in many samples. The chi-squared test of independence (likelihood ratio), the Kendall's tau-c and Kruskal-Wallis test were used to investigate possible relations between STEC or *Salmonella, E. coli* and coliforms, respectively, and several categorical (nominal) factors, such as type of produce and the country of origin.

5.3 Results

In the period from February 2013 to January 2014 (+ 14 additional basil samples originating from Cyprus collected during week 24 and 26 in June 2014), 592 samples of fresh pre-packed basil (*Ocimum basilicum*) and coriander (*Coriandrum sativum*) leaves (25 g) originating from Belgium, Israel and Cyprus were analyzed for the presence of *Salmonella*, STEC, generic *E. coli* and coliforms. One hundred samples were analyzed for each type of herb and for each country of origin, except in the case of basil originating from Cyprus only 92 samples could be collected due to adverse weather conditions: in May to June 2013 it was too hot for some weeks to harvest basil while in December 2013 it was too cold which resulted in cold damage (quality defects). Although some sampling was continued in 2014, in order to obtain the scheduled 100 basil samples from Cyprus, difficulties in obtaining herbs were again encountered due to heavy rain fall in May 2014 resulting in quality defects and thus import of basil was stopped. The harvest period of coriander and basil in Belgium lasted from June until September 2013, while import of basil and coriander from Israel was steady the whole year around (February 2013 to January 2014). Table 12 gives an overview of the microbiological results.

Table 12: Overview results of basil and coriander leaves analyzed for the presence of *Salmonella*, STEC, *E. coli* and coliforms.

		Coriander			Basil		
		Israel	**Cyprus**	**Belgium**	**Israel**	**Cyprus**	**Belgium**
Coliforms[a]	**Samples positive**	100/100	100/100	100/100	100/100[b]	92/92	100/100[b]
	≥ 2.0 and < 3.0 log cfu/g	4/100	4/100	0/100	6/100	1/92	14/100
	≥ 3.0 and < 4.0 log cfu/g	12/100	12/100	8/100	13/100	8/92	26/100
	≥ 4.0 and < 5.0 log cfu/g	26/100	31/100	35/100	37/100	26/92	33/100
	≥ 5. log cfu/g	58/100	53/100	57/100	43/100	57/92	26/100
E. coli[a]	**Weeks positive**	4/51	12/37	9/14	8/48	16/24	5/13
	Samples positive	6/100	24/100	18/100	12/100	42/92	6/100
	≥ 1.0 and < 2.0 log cfu/g (CLASS 2)	3/6	13/24	14/18	3/12	18/42	4/6
	≥ 2.0 and < 3.0 log cfu/g (CLASS 3)	2/6	7/24	4/18	7/12	11/42	1/6
	≥ 3.0 log cfu/g (CLASS 4)	1/6	4/24	0/18	2/12	13/42	1/6
STEC (*stx*)		3/100 (2 x *stx1,* 1 x *stx2)*	8/100 (4 x *stx1,* 7 x *stx2)*	1/100 (1 x *stx2*)	2/100 (1 x *stx1,* 1 x *stx2*)	1/92 (1 x *stx1*)	2/100 (1 x *stx1,* 1 x *stx2*)
STEC (*stx* and *eae*)	**Weeks positive**	3/51	4/37	0/14	1/48	1/24	1/13
	Samples positive	3/100	5/100	0/100	1/100	1/92	1/100
STEC (*stx*, *eae* and serotype)		0/3	3/5	NA	0/1	0/1	0/1
Salmonella	**Weeks positive**	0/51	3/37	0/14	2/48	2/24	0/13
	Samples positive	0/100	5/100	0/100	2/100	3/92	0/100

[a]Detection limit = 1.0 log cfu/g; [b] One sample below 2.0 log cfu/g (1.6 cfu/g for basil originating from Israel, 1.8 log cfu/g for basil originating from Belgium)

Salmonella has been isolated from 10 out of 592 (1.7%, 95% C.I. 0.9% - 3.1%) samples. Further serotyping of the *Salmonella* strains resulted in five isolates of *S. enterica* subsp. *salamae* (three from coriander and two from basil, all originating from Cyprus), three isolates of *S.* Thompson (two from basil originating from Israel and one from coriander originating from Cyprus), one *S. enterica* subsp. *diarizonae* from coriander from Cyprus and one *S.* Infantis from basil also from Cyprus (Table 13).

STEC (defined as the simultaneous presence of *stx* and *eae* genes) has been detected by PCR in 11 out of 592 (1.9%, 95% C.I. 1.0% - 3.4%) samples, whereby in 3 out of 11 PCR positive STEC samples a matching serotype (one O26 and two O103 and O111) and *eae*-variant (1 x *eae*-β and 2 x *eae*-θ, respectively) were detected (0.5%, 95% C.I. 0.2% - 1.5%) (Table 14). The three samples were coriander sampled in three different weeks and all originating from Cyprus. In 6 out of 592 samples, *stx* genes were detected without the presence of *eae* genes, while in 269 out of 592 samples, *eae* genes were detected without the presence of *stx* genes. The *aggR* gene was not detected in any sample. No STEC PCR samples could be confirmed by culture, i.e. no STEC isolates were obtained.

E. coli as a fecal hygiene indicator was detected (≥ 1.0 log cfu/g) in 108 out of 592 samples (18.2%, 95% C.I. 15.3% - 21.6%): 55, 32 and 13 samples respectively between 1.0 – 2.0, 2.0 – 3.0 and 3.0 – 4.0 log cfu/g and 8 samples exceeding 4.0 log cfu/g (Figure 22). Coliforms were, as expected, enumerated in every sample analyzed (Figure 23).

In 6 out of 11 and 2 out 10 samples positive for STEC (by PCR) and *Salmonella* (by culture) respectively, no *E. coli* could be enumerated (i.e. *E. coli* numbers are < 1.0 log cfu/g) (Table 13, Table 14). However, in all 3 samples, that showed the presence of *stx* genes, the serotype O103, O111 or O26 and the most likely associated *eae* gene variants (θ or β), *E. coli* was enumerated (Table 14).

The number of samples taken each week per herb and per country of origin depended on the availability of the herbs in the trading company (Figure 21). In total, *E. coli* has been enumerated in 31 sampling weeks (basil 22 weeks, coriander 19 weeks). In 14 out of 31 sampling weeks (basil 9 out of 22; coriander 7 out of 19) all samples taken in that week were contaminated with *E. coli*, ranging from 1.0 to more than 4.0 log cfu/g (Figure 22).

Table 13: Overview of herb samples positive for *Salmonella.*

Year/Week	*Salmonella*	Country	Herb	*E. coli* (log *cfu/g)*	Coliform (log cfu/g)
2013/w16	*S. enterica* subsp. *enterica* serovar Thompson	Israel	Basil	< 1.0	4.6
2013/w22	*S. enterica* subsp. *diarizonae* serovar 50:I,v:e,n,x,z_{15}	Cyprus	Coriander	2.5	6.0
2013/w27	*S. enterica* subsp. *enterica* serovar Infantis	Cyprus	Basil	3.9	6.4
2013/w27	*S. enterica* subsp. *salamae* serovar 42:b:e,n,x,z_{15}	Cyprus	Coriander	3.2[a]	6.6
2013/w27	*S. enterica* subsp. *salamae* serovar 42:b:e,n,x,z_{15}	Cyprus	Coriander	2.8	6.3
2013/w27	*S. enterica* subsp. *enterica* serovar Thompson	Cyprus	Coriander	3.0	7.0
2013/w28	*S. enterica* subsp. *salamae* serovar 4,[5],12:b:-d-Tartraat	Cyprus	Coriander	2.6	6.2
2013/w48	*S. enterica* subsp. *salamae* serovar 40:z_{39}:z_4,z_{24}	Cyprus	Basil	2.6	6.0
2013/w48	*S. enterica* subsp. *salamae* serovar 40:z_{39}:z_4,z_{24}	Cyprus	Basil	1.8	5.5
2013/w50	*S. enterica* subsp. *enterica* serovar Thompson	Israel	Basil	< 1.0	5.8

[a]: also STEC detected by PCR

Table 14: Overview of herb samples PCR positive for STEC.

Year/Week	STEC (PCR)	Country	Herb	*E. coli* (log cfu/g)	Coliform (log cfu/g)
2013/w13	*Stx1, eae*	Israel	Coriander	< 1.0	5.3
2013/w18	*Stx2, eae*	Israel	Coriander	< 1.0	5.9
2013/w24	*Stx2, eae*	Israel	Basil	< 1.0	4.6
2013/w27	*Stx1, eae*-β, O103	Cyprus	Basil	3.8	6.3
2013/w27	*Stx1, eae*-θ	Belgium	Basil	< 1.0	3.5
2013/w27	*Stx2,* ***eae*-θ***, eae*-β, O145, **O111**, **O103**	Cyprus	Coriander	3.2[a]	6.6
2013/w40	*Stx1,* ***eae*-β**, **O26**	Cyprus	Coriander	1.5	5.1
2013/w47	*Stx1, stx2, eae,* O111	Cyprus	Coriander	< 1.0	4.7
2013/w47	*Stx1, stx2,* ***eae*-θ**, O145, **O111**, **O103**	Cyprus	Coriander	1.0	5.2
2013/w48	*Stx2, eae*-θ, *eae*-β, O145	Cyprus	Coriander	1.7	5.7
2014/w4	*Stx1, eae*	Israel	Coriander	< 1.0	4.8

[a]: also *Salmonella* isolated

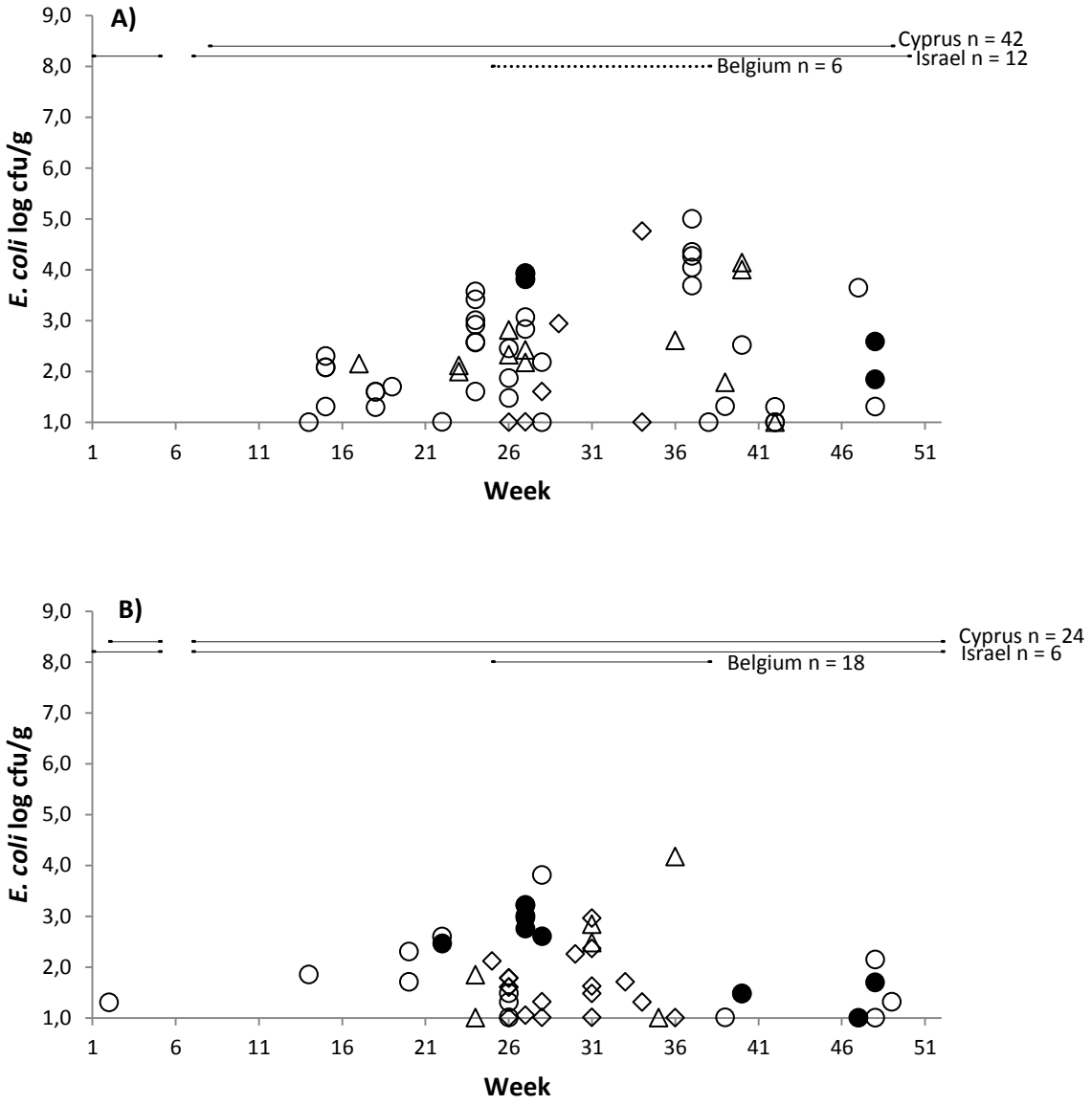

Figure 22: Distribution of *E. coli* (log cfu/g) enumerated on basil (A) and coriander (B) originating from Belgium (◇), Israel (○) and Cyprus (△)(detection limit 1.0 log cfu/g). Closed symbols indicate samples positive for *Salmonella* and/or STEC (presence of *stx* and *eae*). The sampling period is indicated above the figure (n = number of samples positive for generic *E. coli*). One basil sample originating from Cyprus (week 37) exceeded countable numbers (TNTC) and was not displayed.

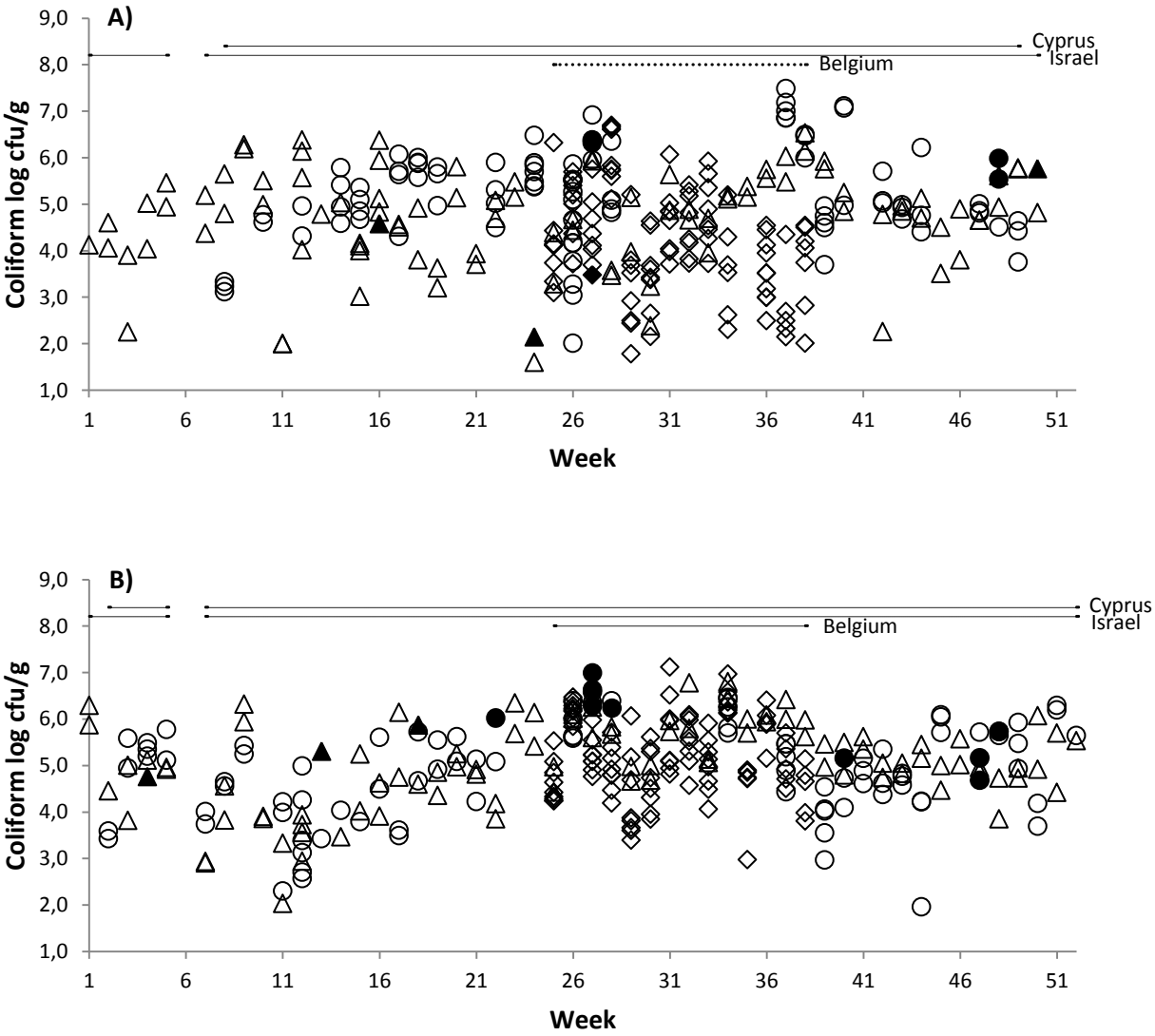

Figure 23: Distribution of coliforms (log cfu/g) enumerated on basil (A) and coriander (B) originating from Belgium (◇), Israel (○) and Cyprus (△) (detection limit 1.0 log cfu/g). Closed symbols indicate samples positive for *Salmonella* and/or STEC (presence of *stx* and *eae*). The sampling period is indicated above the figure.

Further statistical analysis resulted in a significant difference in coliform counts between samples positive and negative for *Salmonella* (Mann-Whitney U Test, $p < 0.001$), but not for STEC (Mann-Whitney U Test, $p = 0.405$). On the other hand, the presence of *Salmonella* (Kendall's tau-c, $p < 0.001$) or STEC (Kendall's tau-c, $p = 0.019$) was significantly correlated with the observed contamination level of *E. coli*, thus the presence of *Salmonella* or STEC in a sample was significantly associated with a higher *E. coli* count in that sample (Figure 24). The prevalence of *Salmonella* was significantly higher for Cyprus in comparison to Belgium (Chi^2, Likelihood ratio, $p = 0.003$), whereas no difference between Belgium and Israel (Chi^2,

Likelihood ratio, p = 0.499) and Israel and Cyprus (Chi², Likelihood ratio, p = 0.058) was found. The prevalence of STEC (Chi², Likelihood ratio, p = 0.132) was not significantly different for the different countries. No significant difference was found for generic *E. coli* counts between the countries (Kendall's tau-c, p = 0.600), in contrast to coliform counts which were significantly higher for Cyprus in comparison to Belgium (Mann-Whitney U Test, p < 0.001), but did not differ between Belgium and Israel (Mann-Whitney U Test, p = 0.062) and Israel and Cyprus (Mann-Whitney U Test, p = 0.059). Lastly, the prevalence of *Salmonella* (Chi², Likelihood ratio, p = 1.000) or STEC (Chi², Likelihood ratio, p = 0.223) and counts for *E. coli* (Kendall's tau-c, p = 0.091) did not differ significantly between basil and coriander. Only coliform counts were significantly higher for coriander compared to basil (Mann-Whitney U Test, p < 0.001).

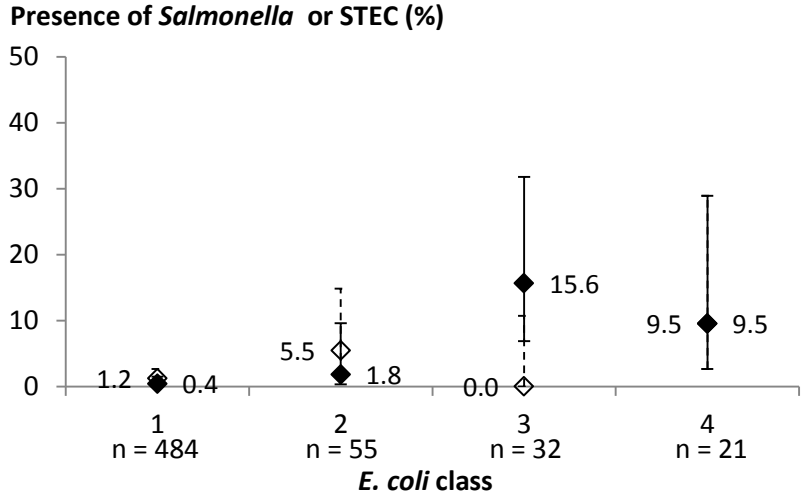

Figure 24: Correlation between the presence of *Salmonella* (full symbols) or STEC (detection by PCR; open symbols)) and the generic *E. coli* class (class 1: undetected, class 2: 1.0 – 2.0 cfu/g, class 3: 2.0 – 3.0 cfu/g, class 4: ≥ 3.0 cfu/g). Flags indicating 95% C.I.

5.4 Discussion

Leafy greens, including fresh herbs, are considered as the highest priority (level 1) in terms of microbiological hazards as they are grown and exported in large volume, have been associated with foodborne outbreaks and are grown and processed in diverse and complex ways (FAO/WHO, 2008; Pakalniskiene et al., 2009; Pezzoli et al., 2008). Moreover, fresh herbs are mostly consumed raw as a decoration or seasoning, whereby, if contaminated with pathogens, an inactivation step is lacking. The sampling plan was set up to get approximately the same number of samples from the various sourcing regions in order to

have similar (un)certainty on the estimates of the prevalence of pathogens and overall microbiological quality established by enumeration of indicator organisms for the three sourcing regions. In the present study, *Salmonella* has been isolated from 5 out of 292 and 5 out 300 basil and coriander samples, respectively, from the Belgian retail. Aytac et al. (2010) and Elviss et al. (2009) reported the presence of *Salmonella* on basil in Turkey (1 out of 8) and the UK (9 out of 674), while Elviss et al. (2009), Singh et al. (2007) and (Quiroz-Santiago et al. (2009) reported the presence of *Salmonella* on coriander in the UK (3 out of 733), North India (9 out of 304) and Mexico (11 out of 100), respectively. On the other hand, no *Salmonella* was found on coriander in Washington D.C. (0/10) (Thunberg et al., 2002), in Saudi-Arabia (0/15) (Al-Holy et al., 2013), in Ontario (0/61) (Arthur et al., 2007) and in southern United Staes (0/94) (Johnston et al., 2005). Samples of previous mentioned studies were either obtained from retail or sampled from the field. However, due to the low number of samples analyzed in the previous studies, the uncertainty on the outcome of the estimated prevalence is large, especially when only 10 or 15 samples were analyzed. Nevertheless, the prevalence of *Salmonella* (1.7 %) in the present study is much lower in comparison to the prevalence of *Salmonella* on imported fresh herbs from South East Asia in Norway in 2005 and 2007, 28% and 15% respectively (NSCFS, 2008). In the present study, three different *Salmonella enterica* subspecies were confirmed: *enterica* (serovar Thompson & Infantis), *salamae* and *diarizonae*. The subspecies *salamae* and *diarizonae* and the serovar Infantis have been already isolated from dried mint, anise and coriander, respectively (Sagoo et al., 2009; Van Doren et al., 2013), while *S.* Thompson has already been linked with a cilantro outbreak in Southern California (Campbell et al., 2001).

Detection of STEC on fresh herbs has only been described a few times, usually involving culture methods for the isolation of *E. coli* O157 only (Al-Holy et al., 2013; Arthur et al., 2007; Johnston et al., 2005). In none of the previous studies, *E. coli* O157 has been isolated from coriander (0/94, 0/61 and 0/15, respectively). However, non-O157 STEC may also cause severe disease (HUC and HC), but the isolation of non-O157 is hampered due to the lack of differentiating biochemical or physicochemical characteristics which are unique for these pathogenic *E. coli*. This leads to insufficient performance of current methods to pick up isolates of STEC (Brooks et al., 2004; EFSA, 2013c). Therefore, the use of molecular detection methods, such as PCR, are preferred as STEC are mainly distinguishable from commensal *E.*

coli on a genetic basis. In the present study, eight coriander and three basil samples were potentially contaminated with pathogenic STEC due to the presence of *stx* and *eae* genes. Three out of these eight coriander samples harbored an STEC with an increased pathogenic potential, namely serotypes O103, O111 or O26 from the "top five" of the EU (O157, O26, O111, O103 and O145) with the most commonly associated *eae*-variant (Bibbal et al., 2014; EFSA, 2013c; Madic et al., 2010; Oswald et al., 2000; Posse et al., 2007). However, no isolates were obtained. Due to the prior enrichment step, PCR detection of DNA from dead cells is less likely, still interpretation and public health implications of detection of these virulence genes is debatable (Ceuppens et al., 2014). But difficulties to obtain actual STEC isolates after PCR detection of *stx* and *eae* genes have been reported before in other STEC studies (Bettelheim, 2003; Delbeke et al., 2015; Joris et al., 2011b). In contrast, Feng & Reddy (2013) reported the presence of an STEC O26 isolate (*stx1*, *eae*-β) on cilantro. The *aggR* gene, described in the *E. coli* O104:H4 outbreak (Rasko et al., 2011), was not detected in the present study in any of the samples, nor in the study of Feng & Reddy (2013) describing 132 STEC isolates from fresh produce, indicating that the presence of *aggR* genes is rather exceptional.

Specific detection of pathogens in food is expensive and time consuming. As the prevalence of pathogens such as *Salmonella* or STEC in fresh herbs is generally below 1-2 %, the cost-effectiveness of random testing is low. It has been suggested that testing of leafy greens (and in this case study thus fresh herbs) for *Salmonella* itself could be limited to instances where other factors indicate poor agricultural or processing practices and thus increased probability for pathogen contamination (EFSA, 2014c). *E. coli* originates from the intestinal tract of man and warm-blooded animals. Hence, the presence of *E. coli* in food indicates that fecal contamination occurred and that consumers might be exposed to enteric pathogens. Therefore, *E. coli* can be used as an indicator organism, i.e. fecal contamination occurred. Coliforms as indicator organism are less accurate, as the group contains species which are not of fecal origin, but are normal inhabitants of plants (e.g. fresh produce) and the environment (FAO/WHO, 2008; Johnston et al., 2005; Mossel et al., 1995; Tortorello, 2003). No hygiene criteria of *E. coli* for fresh herbs exist at primary production phase although an *E. coli* Process Hygiene Criterium for pre-cut fruit and vegetables is set within EU in which a threshold value for marginal quality is set at 100 cfu/g and for unacceptable sanitary quality

at 1000 cfu/g (EC/2073/2005). In the present study, *Salmonella* and/or STEC were detected in 3 out of the 21 samples exceeding the *E. coli* threshold value for unacceptable sanitary quality. Applying the guidelines described by the Public Health England (PHE) for ready-to-eat (RTE) food placed on the market (< 20 cfu/g satisfactory; 20 - $\leq 10^2$ cfu/g borderline; > 10^2 cfu/g unsatisfactory) resulted in 53 samples that were unsatisfactory. Of these samples, 8 were contaminated with *Salmonella* and/or STEC. Also according to (ICMSF, 2011) the end product of fresh and fresh-cut vegetables is unsatisfactory when *E. coli* exceeds 10^2 cfu/g. Thus, a lower *E. coli* threshold value for unacceptable sanitary quality results in a higher chance of detecting *Salmonella*, but also in more samples that are unsatisfactory where no pathogens were detected and thus leading to economic losses and food waste. In addition, in 8 herb samples positive for *Salmonella* and/or STEC, no *E. coli* was enumerated (below detection limit of 1.0 log cfu/g). But, whenever a STEC serotype and its associated *eae*-variant was detected, *E. coli* was also enumerated. In Elviss et al. (2009) 55.6% of the fresh herbs samples, which were positive for *Salmonella*, were negative for *E. coli* (below detection limit of 20 cfu/g). Orozco et al. (2008) and Endley et al. (2003) reported that no *Salmonella* positive samples (tomato and carrots, respectively) showed the presence of *E. coli* and/or only low numbers of coliforms. In Sagoo et al. (2003), *E. coli* was absent (< 20 cfu/g) in three out of the five *Salmonella* positive RTE salad vegetables from retail in UK. The other *Salmonella* positive samples contained 20 and 620 *E. coli* cfu/g. These findings indicate that elevated levels of *E. coli* are related to a failure of good agricultural or hygienic practices but although a significant relation between increased *E. coli* levels and the presence of *Salmonella* in fresh herbs (Kendall's tau-c $p < 0.001$) was established in the present study, the predictive value of elevated levels of *E. coli* for the presence of *Salmonella* is generally low. Coliforms were detected in all herb samples and in variable levels indicating that coliforms are an unsuitable indicator organism for fecal contamination of fresh herbs. Also Al-Holy et al. (2013), Aycicek et al. (2006) and Johnston et al. (2005) reported the presence of coliforms in all investigated leafy greens including coriander, parsley, dill and lettuce.

From this study we can derive that, herbs originating from Cyprus were more frequently associated with generic *E. coli* and *Salmonella* in comparison to the other investigated countries. In Belgium, basil and coriander are mostly grown in greenhouses (in soil), whereas in Cyprus and Israel in open field (in summer) or plastic tunnels (in winter), making these

commodities more vulnerable for contamination, e.g. through wild and domestic animals or insect (Beuchat, 2002; Gil et al., 2015).

Results indicate that fresh pre-packed herbs' leaves such as basil and coriander sampled in a Belgian trading company may pose a potential risk for human health in Belgium. However, the prevalence is low (± 1.6%) and throughout the year-around sampling only during a few weeks samples didn't comply with good microbiological quality and safety. Moreover, no foodborne outbreaks associated with the consumption of fresh herbs were yet reported in Belgium. Still, this may be due to lack of notification, but also whether the presence of *Salmonella* leads to foodborne infection will depend upon the pathogenicity of the strain, the numbers present at the time of consumption and the immunity of the host (consumer). Generic *E. coli* classes are a better indicator for the presence of enteric pathogens than coliforms on fresh herbs, but the relationship between *E. coli* and *Salmonella* or STEC was not strong enough to provide a threshold value to assure food safety (i.e. no pathogens present), as illustrated by the fact that no *E. coli* or low levels of *E. coli* (< 10^2 cfu/g) were present in 3 out of 10 samples which contained *Salmonella*. Differences in microbiological quality and presence of pathogens between sourcing regions/countries may reflect differences in agricultural production and management practices at the farm. It is thus preferred to combine sampling with additional information obtained from audit reports or observational surveys of good agricultural/hygienic practices. In addition, the prevalence of fresh produce might be impacted by seasonality, e.g. higher temperature, rainfall and increased wild life activity in summer might impact on routes of transmission of pathogens and also *E. coli* is more likely to be detected (Holvoet et al., 2015; Rodrigues et al., 2014). However, all samples from the different sourcing regions in the present study were taken in the periods of the year where the climatic conditions were in that way similar. The actual sampling achieved deviates from the optimum sampling plan, which resulted in a different number of samples taken per week. Multi-sampling within a batch will increase the likelihood of finding pathogens in that batch. However, homogenous distribution of pathogens and also fecal indicator organism throughout a batch of herbs is not expected as pathogen's contamination in fresh produce has often been referred to as a point contamination on a field (Hoorfar et al., 2014; Strawn et al., 2013). But, it is acknowledged that multi-sampling within a batch, may lead to some clustered results and impact the final

prevalence estimate. Testing is always restricted in the number of samples that can be analyzed and thus will only detect major non-compliances in good practices, but as mentioned before in the case study of sampling at food service operations (Lahou et al., 2014), also in the present case study for the fresh herbs trading company, sampling and testing for *E. coli* numbers can provide objective results and reveals some information on the quality and safety of the products which cannot be visually checked or necessarily deduced from customer complaints and thus contributes to the verification of supplier selection. An additional benefit of performing some sampling is that it may put pressure on the actual production units at the farmers in the sourcing countries to evaluate and improve their own food safety management system. In addition, data collected by the trading company can be used to build a track record of product control and enable some trend watching over time to verify its own GMP and HACCP plan in ensuring delivery of safe food products to retail and consumers at the end.

CHAPTER 6: Risk ranking of enteric pathogens *Salmonella* and Shiga toxin-producing *Escherichia coli* on fresh basil, strawberries and butterhead lettuce

Redrafted from:

Delbeke, S., Ceuppens, S., Johannessen, G., Allende, A., Sampers, I., Jacxsens, L. & Uyttendaele, M. Risk ranking of enteric pathogens *Salmonella* and Shiga toxin-producing *E. coli* on fresh basil leaves, strawberries and butterhead lettuce. (In preparation)

ABSTRACT

Risk ranking can be used as a tool to set priorities, assisting decision makers to focus on the most significant human health-related problems. Risk ranking in the present context of microbial food safety of fresh produce is used as an approach in defining the relative risk of a number of pre-defined pathogen/commodity combinations. Data related to the prevalence of *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC) on basil, strawberries and butterhead lettuce and these pathogens' behavior during (home) storage were collected by dedicated sampling plans and lab analysis. Information about consumer behavior, i.e. storage time and temperature, if and how washing occurred before consumption and consumption frequency and volumes were collected by a dedicated consumer survey. The effect of washing practices on reduction of pathogen contamination was derived from literature. The quantitative exposure assessment model was built in MS Excel with @Risk software and run by Monte Carlo simulations. The probabilistic output of the model resulted in a distribution of the number of contaminated portions that consumers are exposed to per year. The highest number of contaminated portions was found for basil, followed by lettuce and strawberries. This is particular due to the observed higher prevalence of enteric pathogens on basil leaves, their better survival during storage and less occurrence of consumers washing the basil leaves before consumption. It was calculated that daily consumption of basil can lead to exposure of five to nine contaminated portions a year, both in the Belgian and Spanish situation. The lowest exposure was observed for strawberries in the Spanish situation mainly due to the lower consumption frequency of strawberries but in combination also with higher die-off of the enteric pathogens on strawberries versus basil leaves or lettuce. From the scenario analysis it was noted that the main driving force in exposure to contaminated portions is the prevalence of pathogens in these fresh produce items, a moderate effect of consumption frequency and to a minor extent the consumer behavior (such as time/temperature of storage or washing practices). This quantitative risk ranking calculation demonstrates that also niche products such as basil leaves can have an impact on public health equal or higher than lettuce and also need priority in food safety protection, monitoring and surveillance whereas strawberries indeed are confirmed to be quite safe fruit items.

6.1 Introduction

Salads and various lettuce varieties such as baby leaves have already been long widely acknowledged to be top priority in ensuring in food safety of fresh produce (FAO/WHO, 2008). The question arises to which extent also niche products with minor consumption frequency or volumes such as basil leaves or products usually assumed to be safe such as strawberries might also represent a relevant food safety risk for enteric bacterial pathogens such as *Salmonella* and Shiga toxin producing *E. coli* (STEC).

In the present study, commodities and pathogens of interest were predefined as being fresh herbs and strawberries and *Salmonella* and STEC. The objective was thus to initiate a quantitative risk ranking for basil and strawberries and considering lettuce in the present study as a reference item to assess how these niche products, but increasingly popular in culinary preparations in the West-European diet, rank in reference to lettuce. A comparative retail to fork exposure assessment model was built to investigate the effect of storage time and temperature, washing practices prior to consumption, and consumption frequency exemplified from using a Western European (based on Belgian consumption information) or Mediterranean diet (based on Spanish consumption information). The exposure assessment models incorporated specific data collected on the prevalence of *Salmonella* and STEC on basil, strawberries and butterhead lettuce in Belgium and Norway within the framework of the EU FP7 Veg-i-Trade project as well as data on consumer handling practices and consumption frequency and volumes via a survey in Belgium and Spain (Jacxsens et al., 2015). Taking the measured prevalence of the pathogens into account, a risk ranking, based on the output of the exposure assessment model, is made aimed at comparing pathogens, commodities or regions within Europe (Belgium as an example of a North-western European country versus Spain as an example of a Mediterranean country) in their potential vulnerability towards these microbiological hazards related to the consumption of these three fresh produce items in the diet.

6.2 Materials and Methods

The exposure assessment model from the point of retail to consumption of fresh produce, including purchase, storage by consumer, washing practices of consumers and their consumption, was constructed in MS Excel and run three times by Monte Carlo simulations in @Risk (version 6, Palisade, USA) software (100,000 iterations) (Figure 25).

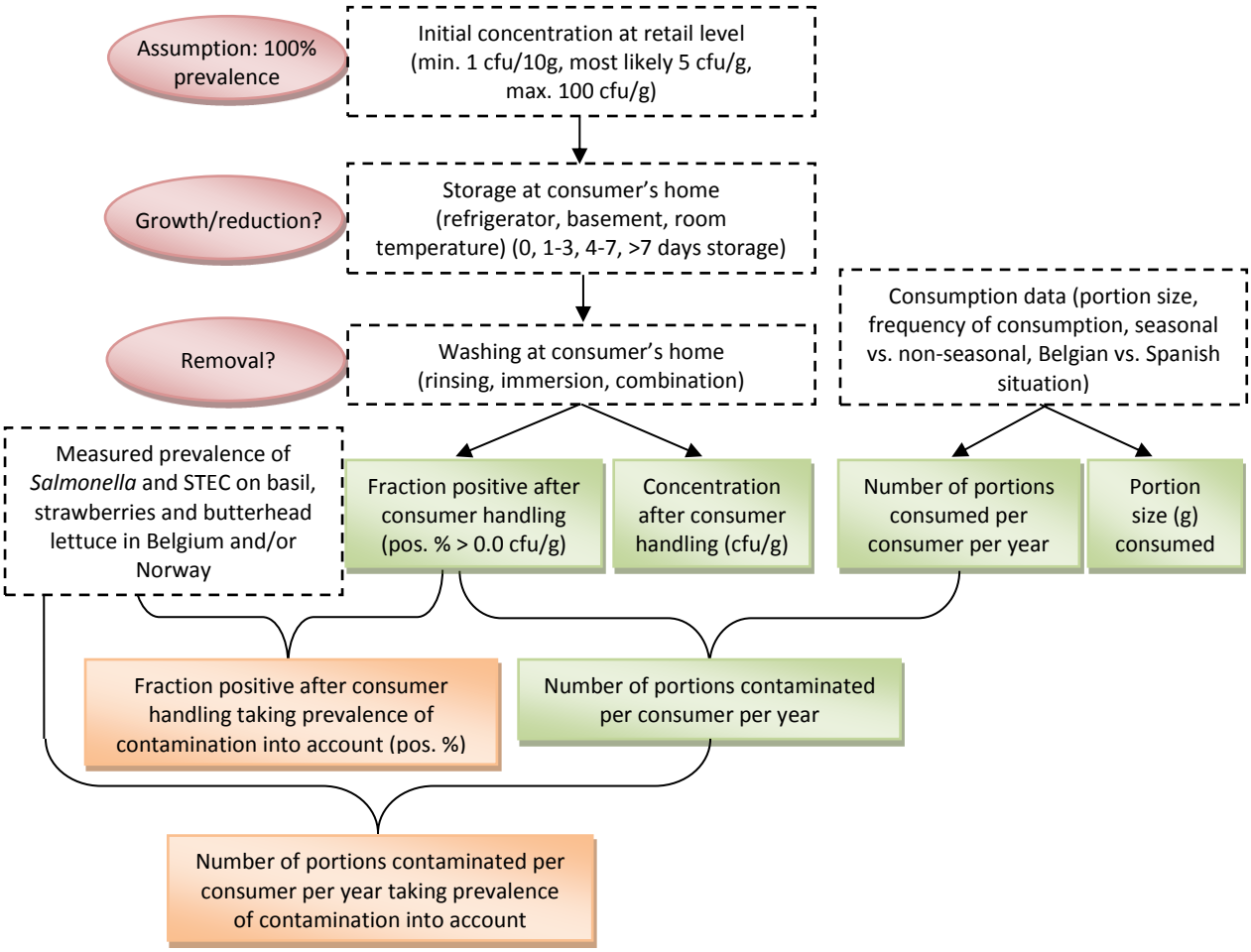

Figure 25: Schematic overview of included modules of retail to fork exposure model for basil, strawberries and butterhead lettuce (green and orange boxes represent the intermediate and finale outputs, respectively)

In the beginning of the model, a 100% prevalence of *Salmonella* and STEC was assumed to determine the influence of storage and washing on the survival/growth/reduction and the removal of these pathogens on basil, strawberries and lettuce, respectively. Simulating the model resulted in a distribution describing the concentration of *Salmonella* and STEC on the commodities after storage and washing. From this distribution, it could be derived that in a

certain fraction of the commodities no pathogens were present anymore (negative fraction = concentration equal to 0.0 cfu/g). Moreover, a certain fraction was assumed to be spoiled due to longer storage time applied by the consumers than could be reasonably foreseen as derived in our lab experiments. This was inserted in the modelling as 10^6 cfu/g, to be able to differentiate from the non-spoiled. The spoiled fraction was further derived by calculating the percentile where the value of the concentration after storage becomes > 10^6 cfu/g and subtracting this percentile from 100%. Subsequently, the positive fraction after storage and washing could be calculated by subtracting the spoiled and negative fraction from 100%. Combining the positive fraction, the prevalence of *Salmonella* or STEC on basil, strawberries or lettuce and the number of portions consumed per year in the Belgian or Spanish situation, gives the number of portions contaminated per year for a certain pathogen/commodity combination. The latter was used to make the ranking of the pathogen/commodity combinations.

The model needed input data related to:

(i) the prevalence and concentration of *Salmonella* and STEC on basil, strawberries and butterhead lettuce,
(ii) the behavior (growth, survival, reduction) of these enteric bacterial pathogens on these considered fresh produce items during (home) storage,
(iii) the potential removal of these pathogens by prior washing procedures before consumption,
(iv) consumer behavior with relation to storage and washing handling and consumption.

This information was collected by making use of i) data collected within the EU FP7 Veg-i-Trade project on *Salmonella* and STEC prevalence by sampling and analysis of a) strawberries and butterhead lettuce at the production sites, i.e. at farms in either Norway or Belgium, or at retail, processing or trading level in Belgium and of b) fresh-cut basil leaves to be put on the market in Belgium, (ii) in house laboratory experiments to assess the behavior of *Salmonella* and STEC on basil leaves, intact strawberries or lettuce leaves (CHAPTER 3), (iii) a consumer survey in Spain and Belgium, performed within the EU FP7 Veg-i-Trade project, on consumer food handling practices and consumption of basil, strawberries and lettuce

(Jacxsens et al., 2015) and further complemented with (iv) literature data and v) assumptions, if still needed, based on authors' expertise and rationale. For example, it is assumed that prevalence (and concentrations) of pathogens does not change between sampling at farm or at trade and transport to the auction/wholesale or retail and during transport from retail to consumer's home. Usually time for transport and storage between harvest, packing and sale in Europe is quite short (e.g. in Belgian within the range of 24 to maximum 72 hours) and under proper temperature control (< 4°C for butterhead lettuce and strawberries and at ca. 10 °C for fresh-cut basil leaves). Also time at retail and time from retail to consumer's home is expected to be quite short (e.g. in the Netherlands 7.9 ± 5.9 min, in the UK 42.8 ± 18.7 min (Nauta, 2001)) and thus assumed not to impact on prevalence and concentrations of the enteric pathogens under consideration.

6.2.1 Input data collection

6.2.1.1 Prevalence and concentration of *Salmonella* and STEC on basil, strawberries and butterhead lettuce

Presence of *Salmonella* and STEC in 25 g (or 60 g in case of strawberries) was determined during the sampling and analysis in the framework of the EU FP7 project Veg-i-Trade and is in detail described in CHAPTER 4 (n = 72 for strawberries at primary production in Belgium), CHAPTER 5 (n = 292 for basil leaves at trading level in Belgium) and in Araujo Fernandes (2014) and Holvoet et al. (2014) (n = 126 for butterhead lettuce at primary production in Belgium). In addition, unpublished data collected during the EU FP7 project Veg-i-Trade (n = 105 for strawberries at retail and trading level in Belgium and n = 80 for strawberries and n = 153 for butterhead lettuce at primary production in Norway) was included. From the data collected in these surveys, the prevalence of *Salmonella* and STEC in basil leaves, strawberries and butterhead lettuce is determined (Table 15).

The GeneDisc Cycler (Pall GeneDisc, France) was used for the detection of *Salmonella* and STEC by real-time PCR in 25 g for leafy greens and in 60 g for strawberries, whereby it was screened for the *iroB* gene and *stx1*, *stx2* and *eae* gene, respectively. Subsequently, positive PCR samples were confirmed. For *Salmonella*, ISO 6579:2002 (Belgium) or NMKL method (Norway) was used, whereas in the case of STEC, ISO 16654:2001 in parallel with plating on CHROMagar STEC (CHROMagar Microbiology, France) or immunomagnetic separation of

non-O157 serotypes and plating. Presumptive colonies were further characterized by biochemical and/or serological methods.

Table 15: Overview of prevalence data of *Salmonella* and presumptive STEC on basil leaves, strawberries and butterhead lettuce obtained during sampling and analysis in the framework of the EU FP7 project Veg-i-Trade.

	Salmonella	**STEC[a]**
Basil	5/292 (95% C.I. 0.7 - 3.9)[b]	3/292 (95% C.I. 0.4 - 3.0)[b]
Strawberries	0/257 (95% C.I. 0.0 - 1.5)[b]	0/257 (95% C.I. 0.0 - 1.5)[b]
Butterhead lettuce	0/279 (95% C.I. 0.0 - 1.4)[b]	0/279 (95% C.I. 0.0 - 1.4)[b]

[a]: *stx* and *eae* genes detected by PCR (culture confirmation was not successful) = presumptive positive for STEC; [b]: the 95% Confidence Interval (C.I.) was calculated according to E.B. Wilson (Wilson, 1927).

Furthermore, to build up the exposure assessment model, a distribution of the initial concentration for the positive samples is needed as an input to determine the effect of storage and of washing practices and thus the calculation of the residual concentration of the pathogens on the commodities. An assumption based on expert discussion was made resulting in a minimum value of 1 cfu/10 g, a most likely value 5 cfu/g and a maximum value of 100 cfu/g, as these fresh produce items are mostly contaminated with a low number of cells of these enteric pathogens (Pielaat et al., 2008). In Danyluk & Schaffner (2011), a starting level of 1 cfu/10 g was used for the quantitative assessment of the microbial risk of leafy greens. It should be noted that the same prevalence of *Salmonella* and STEC was assumed in the Belgian and Spanish situation.

6.2.1.2 The behavior of *Salmonella* and *E. coli* O157:H7 on the considered fresh produce items during (home) storage

The survival of *Salmonella* Thompson and Typhimurium and *E. coli* O157:H7 was investigated on basil, strawberries and butterhead lettuce by artificial inoculation of these commodities and storage at different temperature (CHAPTER 3). The initial concentration (day 0) was not considered because after inoculation on fresh produce, the pathogens need to adapt to the new environment. Subsequently, log reductions per day were calculated by reference to day 1 (= average log cfu/g day 1 – log cfu/g day x) and, when no significant difference was found,

results were grouped together (strawberries at 4°C and 10°C, see Table 16). When the die-off or growth of pathogens was rather limited (average log reductions per day around the measurement uncertainty of plate counts being ca. 0.5 log units (Jarvis et al., 2007)) an increase/decrease of 0 cfu/g during storage was inserted in the model. Otherwise, a (logarithmic) trend line was fitted on the average log reductions and the equations obtained were inserted in the model to describe die-off or growth during storage at consumer's home. The end of shelf life (e.g. due to growth of molds (*Botrytis cinerea*) on strawberries or other (visual) observations of quality decay on basil and lettuce) at a certain time-temperature combination was considered. It was inserted in the modelling as 10^6 cfu/g, to be able to differentiate from the non-spoiled and consequently consumed produce during the simulation of the model (Table 16).

Table 16: Die-off or growth of *E. coli* O157:H7 and *Salmonella* (cfu/g) on basil, strawberries and butterhead lettuce in function of the time (x) (during storage at different temperatures), taking the end of shelf life into account (= -10^6). When no significant difference between temperatures (determined using the non-parametric Kruskal Wallis test in SPPS version 22 with a significant level of 5%) was observed, data was taken together to derive the logarithmic function.

	Storage temperature	Shelf life (days)	Function	
			E. coli O157:H7 (log cfu/g)	*Salmonella* (log cfu/g)
Basil	7°C	10	0	0
	15°C	10	0	0
	22°C	9	IF(x<=9;0;-10^6)	IF(x<=9;0;-10^6)
Strawberries	4°C-10°C	10	IF(x<1;0;10^(0.6873*ln(x) + 0.5484))	IF(x<1;0;10^(0.9177*ln(x) + 0.496))
	15°C	7	IF(x<1;0;IF(x<=7;10^(0.8912* ln(x) - 0.1056);-10^6))	IF(x<1;0;IF(x<=7;10^(0.9177*ln(x) + 0.496);-10^6))
	22°C	4	IF(x<=4;0;-10^6)	IF(x<1;0;IF(x<=4;10^(0.9177*ln(x) + 0.496);-10^6))
Butterhead lettuce	7°C	7[a]	IF(x<=7;0;-10^6)	IF(x<=7;0;-10^6)
	15°C	5[a]	IF(x<=5;0;-10^6)	IF(x<=5;0;-10^6)
	22°C	3[a]	IF(x<=3;0;-10^6)	IF(x<=3;0;-10^6)

[a]: Assumed shelf life

6.2.1.3 The potential removal of enteric pathogens from leafy greens and strawberries by prior washing procedures before consumption

Data relating to the removal of pathogens by washing was collected from the literature. Procedures that mimic the handling of consumers as much as possible were searched for. This means that only experiments were taken into account that use tap water (or distilled

water) without the addition of components (e.g. sodium hypochlorite), pre-treatment of the produce (e.g. destroying the background microbiota) or simulation of industrial processes. Data was collected for leafy greens (e.g. iceberg lettuce, romaine lettuce, green leaf lettuce, cilantro, parsley) and strawberries and for *E. coli*, STEC, *Salmonella* and *Listeria*. There was a distinction made between rinsing with running water, immersion and the combination of rinsing and immersion. No distinction was made between wash procedures with or without a drying step (e.g. centrifugation to remove excess water) and between immersion with or without scrubbing (agitation), as the differences were non-existent or limited (Davidson et al., 2013). No differentiation was made between the different bacteria that were removed from the different leafy greens by washing (Table 17).

Table 17: Overview of log reduction (log cfu/g) of bacteria (*E. coli*, *E. coli* O157:H7, *Salmonella* or *Listeria*) from leafy greens and strawberries by different washing procedures.

	Leafy greens		Strawberries	
	Log reduction (log cfu/g)	**Reference (Country)**	**Log reduction (log cfu/g)**	**Reference (Country)**
Rinsing	1.2	(Pangloli et al., 2009) (China)		
	2.2	(Pangloli et al., 2009) (China)		
	1.4	(Kilonzo-Nthenge et al., 2006) (USA)		
	0.5	(Rodgers et al., 2004) (USA)		
	0.9	(Rodgers et al., 2004) (USA)		
Immersion	0.7	(Beuchat et al., 1998) (USA)	0.7	(Hung et al., 2010) (France)
	0.5	(Lopez-Galvez et al., 2009) (Spain)	0.4	(Hung et al., 2010) (France)
	0.3	(Baert et al., 2009) (Belgium)	0.9	(Hung et al., 2010) (France)
	0.3	(Baert et al., 2009) (Belgium)	0.6	(Hung et al., 2010) (France)
	0.3	(Baert et al., 2009) (Belgium)	0.8	(Rodgers et al., 2004) (USA)
	0.3	(Baert et al., 2009) (Belgium)	0.9	(Rodgers et al., 2004) (USA)
	0.6	(Stopforth et al., 2008) (USA)		
	1.1	(Stopforth et al., 2008) (USA)		
	0.6	(Stopforth et al., 2008) (USA)		
	1.4	(Stopforth et al., 2008) (USA)		
	1.0	(Stopforth et al., 2008) (USA)		
	1.0	(Stopforth et al., 2008) (USA)		
	0.8	(Akbas & Olmez, 2007) (USA)		
	0.8	(Akbas & Olmez, 2007) (USA)		
	0.6	(Akbas & Olmez, 2007) (USA)		
	0.7	(Akbas & Olmez, 2007) (USA)		
	1.0	(Luo et al., 2011) (USA)		
	0.5	(Hao et al., 2011) (China)		
	0.3	(Keskinen et al., 2009) (USA		
	0.6	(Keskinen et al., 2009) (USA)		
	0.8	(Keskinen et al., 2009) (USA)		
	0.6	(Keskinen et al., 2009) (USA)		
	0.8	(Keskinen & Annous, 2011) (USA)		
	0.4	(Sengun, 2013) (Turkey)		
	0.5	(Sengun, 2013) (Turkey)		
	0.5	(Sengun, 2013) (Turkey)		
	0.6	(Sengun, 2013) (Turkey)		
	1.3	(Beuchat et al., 1998) (USA)		
	0.7	(Pangloli et al., 2009) (China)		
	0.9	(Rodgers et al., 2004) (USA)		
	0.8	(Rodgers et al., 2004) (USA)		
Rinsing and immersion	1.9	(Pangloli et al., 2009) (China)		
	2.4	(Pangloli et al., 2009) (China)		
	1.8	(Kilonzo-Nthenge et al., 2006) (USA)		
	1.9	(Kilonzo-Nthenge et al., 2006) (USA)		
	1.8	(Kilonzo-Nthenge et al., 2006) (USA)		
	1.56	(Beuchat et al., 1998) (USA)		
	1.57	(Beuchat et al., 1998) (USA)		
	1.57	(Beuchat et al., 1998) (USA)		
	1.58	(Beuchat et al., 1998) (USA)		
	1.0	(Beuchat et al., 1998) (USA)		
	1.0	(Beuchat et al., 1998) (USA)		
	1.0	(Beuchat et al., 1998) (USA)		
	0.9	(Beuchat et al., 1998) (USA)		

6.2.1.4 Consumer behavior with relation to storage and handling washing and consumption

Data relating to consumer handling practices of fresh produce, more specifically storage (time and temperature), washing (washing versus non-washing) and consumption (frequency and portion sizes) has been collected in Belgium and in Spain by a consumer survey of respectively 1605 and 583 respondents between 18 and 65 years (Jacxsens et al., 2015). For storage time, a distinction was made between consumption at the same day of purchase, after 1 to 3 days, after 4 to 7 days and after more than 7 days. For storage temperature, a differentiation was made between storage in the refrigerator (< 7°C), in the basement (15°C) and at room temperature (22°C). Results for basil, strawberries and butterhead lettuce are summarized in Table 18.

Table 18: Storage time and temperature and washing practices of basil, strawberries and lettuce by Belgian and Spanish respondents in a consumer survey (modified from (Jacxsens et al., 2015))

		Basil		Strawberries		Lettuce	
		Belgium	**Spain**	**Belgium**	**Spain**	**Belgium**	**Spain**
Storage time % (n)	**Same day**	14 (210)	10 (44)	27 (421)	12 (67)	10 (157)	4 (25)
	After 1 – 3 days	32 (479)	28 (126)	61 (959)	55 (312)	69 (1084)	40 (233)
	After 4 – 7 days	31 (455)	35 (161)	12 (184)	27 (156)	19 (296)	41 (237)
	> Week (= 10 days)	23 (341)	27 (124)	1 (8)	6 (33)	2 (30)	15 (84)
	Total consumers (non-consumers)[a]	1485 (93)	455 (144)	1572 (26)	568 (46)	1567 (34)	579 (31)
Storage temperature % (n)	**Refrigerator[b]**	37 (562)	73 (341)	80 (1273)	95 (536)	93 (1477)	99 (572)
	Basement[c]	6 (87)	18 (86)	5 (80)	2 (13)	5 (85)	1 (5)
	Room temperature[d]	58 (884)	9 (43)	15 (242)	3 (16)	1 (23)	1 (3)
	Total consumers (non-consumers)[a]	1533 (87)	471 (140)	1595 (27)	565 (45)	1585 (38)	580 (30)
Wash practices % (n)	**Washing**	68 (1016)	77 (393)	86 (1355)	96 (562)	98 (1398)	99 (583)
	No washing	32 (487)	23 (115)	14 (217)	4 (21)	2 (24)	1 (7)
	Total consumers (non-consumers)[a]	1503 (92)	508 (94)	1572 (23)	583 (16)	1422 (45)	590 (15)

[a]: non-consumers = respondents that do not consume the commodity; [b]: corresponding with 4°C or 7°C during survival experiments; [c]: corresponding with 15°C during survival experiments; [d]: corresponding with 22°C during survival experiments.

6.2.2 Comparative retail to fork exposure assessment model (basic scenario)

The different steps to build the model, the data inputs used and the outputs defined are presented in Table 19 for the case study of basil leaves contaminated with *Salmonella* and consumed according to data obtained by the Belgian respondents (presenting only the example of washing performed by immersion). Similar simulations and outputs have been performed for the other case studies (strawberries and butterhead lettuce, using prevalence data for STEC, using washing by rinsing and combination of rinsing and immersion, using consumer behavior data obtained for Spanish respondents). This change of input data can easily be done by adapting the functions and/or the value of the variables of the parameters. By simulating the exposure assessment model with 100,000 iterations, the basic scenario was created, whereby the following outputs were obtained: (i) concentration after storage at consumer's home (cfu/g), (ii) concentration after washing (rinsing, immersion or combination) (cfu/g), (iii) contaminated (positive) fraction (with or without taking the prevalence into account), (iv) spoiled fraction (being commodity stored beyond the defined shelf lives in Table 16) and (v) a distribution of the number of contaminated portions in one year (with or without taking the prevalence into account). In order to avoid negative concentrations of bacteria, in each module (step) of the model a correction for negative values (< 0.0 cfu/g) was made. To have insight in the stability of the simulations, three simulations were run per combinations of inputs. The mean and standard deviation was applied in our final resulting tables.

Thus, in total, results were obtained for three fresh produce commodities, two enteric bacterial pathogens, three types of washing practices and two types of European consumer behavior (Belgium exemplifying the North-western European consumer and Spain representing a more Southern European consumer and Mediterranean diet) and were included in this risk ranking exercise using the developed exposure assessment model. This resulted in 36 cases of basic scenarios by applying the input information from Table 15, Table 16, Table 17 and Table 18 in the model.

Table 19: Functions of the different parameters inserted in the comparative retail to fork exposure assessment model for basil contaminated with *Salmonella* and consumed according to data obtained by Belgian respondents (green and orange box' showing the output parameters).

	PARAMETER	Function[c]	Details[c]	Unit
S1	Concentration at retail	RiskPert(a;b;c) with a = minimum value, b = most likely value and c = maximum value	a = 0.1, b = 5, c = 100	cfu/g
		STEP 1: STORAGE AT CONSUMER'S HOME STEP		
S2	Storage time	RiskCumul(a1 ; ax ;{a1 ; a2 ; … ; ax} ;{b1 ; b2 ; … ; bx}) with a = different storage times and b = cumulative proportion of consumers	a1 = 0, a2 = 3, a3 = 7, a4 = 10 b1 = 0.14, b2 = 0.46, b3 = 0.77, b4 = 1	d
S3	Storage temperature (refrigerator)	Probability	= 562/1533	-
S4	Storage temperature (basement)	Probability	= 87/1533	-
S5	Storage temperature (room temperature)	Probability	= 884/1533	-
S6	Decrease/increase during storage at 7° (= refrigerator)	0	-	cfu/g
S7	Decrease/increase during storage at 15° (= basement)	0	-	cfu/g
S8	Decrease/increase during storage at 22° (= room temperature)	IF(S2<=a;0;-10^6)[c] with a = end of shelf life at 22°C	a = 9	cfu/g
S9	Decrease/increase during storage	RiskDiscrete(S6:S8;S3:S5)	-	cfu/g
S10	Concentration after storage	= S1 – S9	-	cfu/g
S11	Concentration after storage (correction for negative values)	= If(S10<0;0;S10)	-	cfu/g
		STEP 2: WASHING AT CONSUMER'S HOME		
S12	Removal by washing (immersion)	RiskTriang(a;b;c) with a = minimum, b = average, c = maximum	Minimum, average[a], maximum of removal by immersion (Table 17)	log cfu/g
S13	Consumer handling (washing)	Probability	= 487/1503	-
S14	Consumer handling (no washing)	Probability	= 1016/1503	-
S15	Decrease by washing	= 10^S12	-	cfu/g
S16	Decrease by no washing	0	-	cfu/g
S17	Decrease after washing	RiskDiscrete(S15:S16;S13:S14)	-	cfu/g

(Continue Table 19)

	PARAMETER	Function[c]	Details[c]	Unit
S18	Concentration after washing	= S11 – S17	-	cfu/g
S19	Concentration after washing (correction for negative values)	= If(S18<0;0;S18)	-	cfu/g
S20	Positive fraction (> 0.0 cfu/g)	Fraction	= 0.82[b]	-
		STEP 3: CONSUMPTION BEHAVIOR		
S21	Consumption frequency (seasonal)	RiskDiscrete({a1;a2;a3;a4;a5;a6};{b1; b2;b3;b4;b5;b6}) with a = consumption frequency and b = number of consumers	a1 = 1.5, a2 = 3, a3 =13, a4 = 39, a5 = 91.25, a6 = 273.75, b1 = 23, b2= 51, b3 = 142, b4 = 91, b5 = 35, b6 =3	portions/year
S22	Consumption frequency (non-seasonal)	RiskDiscrete({a1;a2;a3;a4;a5;a6};{b1; b2;b3;b4;b5;b6}) with a = consumption frequency and b = number of consumers	a1 = 6, a2 = 12, a3 = 52, a4 = 156, a5 = 365, a6 = 1095, b1 = 100, b2 = 240, b3 = 191, b4 = 132, b5 = 67, b6 = 1	portions/year
S23	Consumption frequency	RiskDiscrete(S21:S22;{a1;a2}) with a = number of consumers	a1 = 345, a2 = 731	portions/year
S24	Distribution of contaminated portions per year	= S20*S23	-	Number/year
		STEP 4: PREVALENCE OF *SALMONELLA*		
S25	Prevalence on basil	RiskBeta((a+1);(b-a+1)) with a = number of contaminated samples and b = total number analysed samples	a = 5; b = 292	-
S26	Distribution of contaminated portions per year taking prevalence into account	= S25*S24	-	Portions/year
S27	Positive fraction (> 0.0 cfu/g) taking the prevalence into account	= S25*S20	-	-

[a]: Instead of using the mode, the average of the data was used because there were not enough values in every case to calculate the mode (e.g. rinsing of leafy greens); [b]: The positive fraction was obtained from the @Risk distribution created after storage and washing (= 1 – negative fraction – spoiled fraction); [c]: Input for parameters or variables described in Table 15, Table 16, Table 17and Table 18.

6.2.3 Scenario and sensitivity analysis

The basic scenario resulted in the fraction of portions contaminated (= positive fraction) and a distribution describing the number of contaminated portions in one year as final output. Thereby, consumer behavior, related to storage time and temperature and washing practices, collected in Belgium and Spain was used. Subsequently, different "what if" scenarios were created. Lastly, a tornado plot was developed showing the sensitivity of parameters inserted as a distribution in the basic scenario. The scenario analysis and the tornado plot were only performed for the wash practice "immersion" (= worst case due to least removal as derived from Table 17).

6.2.3.1 Scenario analysis

In the basic scenario, only a certain fraction of the consumers stored the commodities in the refrigerator, according to the conducted survey (Jacxsens et al., 2015). The aim of the first scenario was to determine the influence on the final output if all consumers would store the commodity in the refrigerator (Probability (Storage temperature (refrigeration)) = 1). It should be noted that in case of basil, refrigeration is not recommended due the development of cold damage (= chilling injuries) (Lange & Cameron, 1994). However, during one week storage at 7°C, it was observed in our in house lab experiments that chilling injuries were rather limited, therefore this spoilage of basil was not considered in the model (CHAPTER 3). In the basic scenario, only a fraction of the consumers wash the commodities before consumption, again according to the conducted survey (Jacxsens et al., 2015). In the second scenario, the influence on the final output of either (a) all consumers washing the commodity (Probability (Consumer handling (washing)) = 1) or (b) none of the consumers washing the commodity (Probability (Consumer handling (washing)) = 0) was determined. In both scenarios 1 and 2, the concentration and positive or spoiled fraction will change as the value of a parameter during storage or washing has been adapted. Therefore, an adjusted positive fraction after storage and washing must be derived from the model after simulation and inserted to calculate the final output. The positive fraction is defined as the fraction, containing more than 0.0 cfu/g and less than 10^6 cfu/g (spoiled), of the distribution describing the concentration of the pathogens on the commodities after storage and washing at consumer's home.

The following scenarios were only focusing on basil. In scenario 3, the parameter prevalence of *Salmonella* on basil was changed. Instead of using the overall prevalence (basic scenario (5/292)), the prevalence of *Salmonella* on basil cultivated in different countries (Belgium (0/100) vs. Israel (2/100) vs. Cyprus (3/92)) was inserted, to assess the influence of sourcing basil from different countries, and thus different prevalence's, on the final output.

In the last scenario, the prevalence of STEC (presence of *stx* and *eae* genes, 3/292) was replaced by the prevalence of $STEC^{+}$, defined as the presence (by PCR) of a serotype (O145, O103, O111 or O26) and the mostly likely corresponding *eae*-variant (β, ϒ, θ, ε) in addition to the *stx* gene. In this way, the (im)possibility of the *stx* and *eae* virulence factors originating from one *E. coli* strain is gauged by the match of the *eae* variant and the serotype detected by PCR (CHAPTER 4). In none of the commodities $STEC^{+}$ was detected (0/292). Moreover, no STEC was isolated from the commodities.

6.2.3.2 Sensitivity analysis (tornado plot)

During sensitivity analysis, the minimum or maximum value of a distribution attributed to a parameter in the baseline model is used in the model to see the impact on the output. The initial concentration of *Salmonella* and STEC on the commodities was unknown, but was assumed to be low. To determine the influence of storage and washing on the concentration of the pathogens on the commodities, an initial concentration is needed. This was inserted, after expert discussion, by a RiskPert distribution in the model (Table 19). Secondly, the removal of pathogens from leafy greens, basil and strawberries due to washing was collected from literature and described as a RiskTriang distribution (Table 19). Both distributions were built by using a minimum, most likely or average and maximum value. By creating a tornado plot, the influence of inserting the minimum or maximum value of these parameters, instead of the distribution as in the basic scenario, on the final output, could be determined. Moreover, the parameter which has the major influence on the final output could be derived from the tornado plot (Daelman et al., 2013). The tornado plot was only created for basil contaminated with *Salmonella* in the Belgian situation.

6.3 Results

6.3.1 Basic scenario

Firstly, the stability of the model was determined. Therefore, the model was simulated three times with 100,000 iterations. As results of all three simulations were similar (e. g. Table 20: standard deviation maximum 0.22%), it was decided that 100,000 iterations were sufficient for a stable model.

The influence of storage time and temperature and washing practices in the Belgian and Spanish situation on the presence of *Salmonella* and *E. coli* O157:H7 on basil, strawberries and butterhead lettuce is shown in Figure 26. For basil and butterhead lettuce no difference between *Salmonella* and *E. coli* O157:H7 was observed due to the similar behavior in survival of both pathogens during storage (Table 16). Only in the case of strawberries, pathogen counts (cfu/g) decreased during storage. After storage, a certain fraction was assumed to be spoiled due to longer storage time applied by the consumers than could be reasonably foreseen as derived in our lab experiments. More spoilage (and thus waste because no sufficient quality for human consumption) of basil and strawberries was noticed in the Belgian situation due to a higher fraction of these consumers which are storing the product outside the refrigerator in comparison to the Spanish situation (even though Spanish consumers store their product for a longer time (Table 18)). In case of lettuce, a higher spoilage percentage was observed for the Spanish situation due to the longer storage time in combination with the short (assumed) shelf life (7 days when stored at 7°C) of butterhead lettuce. However, it should be noted that also other varieties of lettuce heads (such as iceberg lettuce) are consumed in Spain, which are presumed to have a longer shelf life than the more fragile variety of butterhead lettuce (FreshPlaza, 2014). In addition, the pathogens on all commodities are partially removed by washing, whereby the highest decrease of pathogens was observed when a combination of rinsing and immersion was used (e.g. for basil contaminated by *Salmonella* and consumed in Belgium a removal of 12.0, 4.5 and 17.5 cfu/g was observed for rinsing, immersion and a combination rinsing and immersion, respectively (P50)).

The positive fraction (fraction of produce containing pathogens) differed the most between the Spanish and Belgian situation in case of strawberries due to the longer storage time (=

more die-off), more storage in the refrigerator and more washing by Spanish consumers (Table 18). For lettuce, it should be noted that the difference in positive fraction between the Belgian and Spanish situation is mainly caused by the higher spoiled fraction due to the longer storage time of lettuce in the Spanish situation (15% store lettuce more than a week = 15% spoiled fraction as 7 days was inserted in the model as end of shelf life at 7°C).

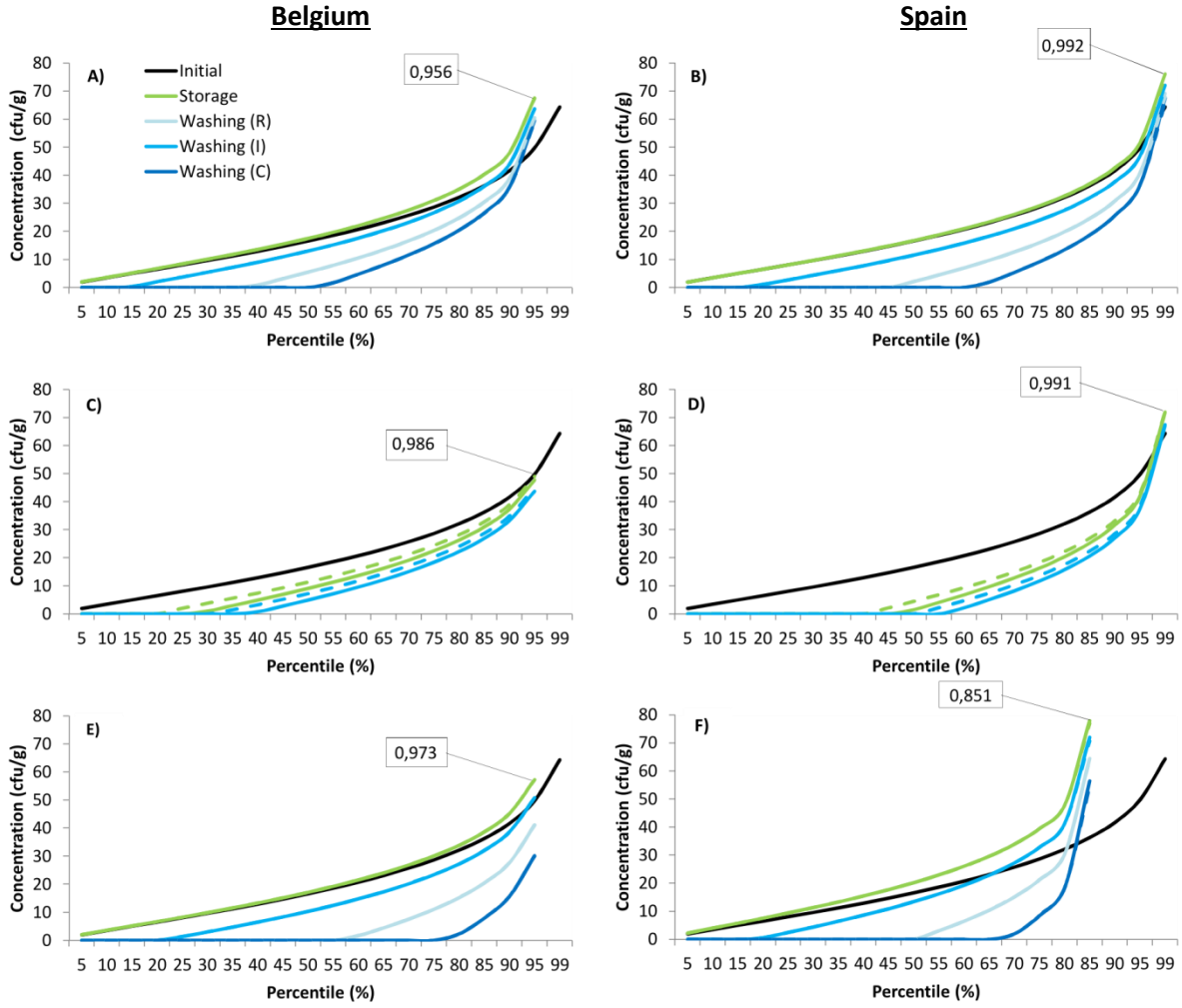

Figure 26: Distributions describing the initial concentration (cfu/g) of *Salmonella* (full line) and *E. coli* O157:H7 (dotted line) on basil (A & B), strawberries (C & D) and lettuce (E & F) and the influence of storage and washing in the Belgian (A, C & E) and Spanish (B, D, F) situation on the counts (n = 3; 100,000 iterations) (R = rinsing, I = immersion, C = combination rinsing and immersion). The value indicates the percentile from which the commodity is spoiled (spoiled fraction = 1 – value). The standard deviation (max. ± 1.46 cfu/g) of the three simulations was not shown in these figures.

The storage method and washing practices were similar in the Belgian and Spanish situation in case of lettuce. Subsequently the positive fraction (Table 20) was used to calculate the distribution of number of samples contaminated per year after storage and washing

assuming that all samples are contaminated at retail (Table 21). This is only shown here for the washing practice 'immersion' (worst case due to lowest removal).

Table 20: Average spoiled fraction of basil, strawberries and butterhead lettuce after storage, which is presumed not to be further consumed anymore by the consumers, and average positive fraction (> 0.0 cfu/g and < 10^6 cfu/g *Salmonella* or *E. coli* O157:H7) after storage and washing in the Belgian and Spanish situation (simulations n = 3; SD = standard deviation) for basic scenario when assuming that the prevalence is 100% at retail.

Produce	Country	Washing	Spoiled fraction		Positive fraction			
			Average	SD	*Salmonella*		*E. coli* O157:H7	
					Average	SD	Average	SD
Basil	Belgium	Rinsing	4.44%	0.03%	57.28%	0.17%	57.22%	0.11%
		Immersion			81.52%	0.11%	81.51%	0.10%
		Combination			43.13%	0.17%	43.07%	0.07%
	Spain	Rinsing	0.83%	0.03%	53.67%	0.09%	53.59%	0.03%
		Immersion			82.44%	0.09%	82.45%	0.07%
		Combination			36.91%	0.03%	36.88%	0.04%
Strawberries	Belgium	Immersion	1.44%	0.01%	60.62%	0.01%	67.49%	0.15%
	Spain	Immersion	0.86%	0.02%	43.21%	0.09%	48.61%	0.03%
Lettuce	Belgium	Rinsing	2.69%	0.01%	40.52%	0.22%	40.49%	0.08%
		Immersion			76.45%	0.09%	76.46%	0.12%
		Combination			19.66%	0.04%	19.80%	0.05%
	Spain	Rinsing	14.89%	0.02%	35.14%	0.18%	35.19%	0.03%
		Immersion			66.75%	0.08%	66.81%	0.11%
		Combination			16.90%	0.04%	16.92%	0.08%

Taking the prevalence of *Salmonella* and STEC on the different commodities into account, the distribution of the number of contaminated samples could be calculated. For butterhead lettuce no difference between both pathogens was observed due to the similar survival behavior and prevalence (no pathogens were detected). Differences between the pathogens on basil and strawberries were assigned to the difference in prevalence and survival behavior, respectively. Number of samples contaminated with pathogens per year were higher in the Spanish situation compared to Belgian situation in case of basil and lettuce due to the more frequent consumption and difference in storage and washing practices (Table 18). The opposite was observed for strawberries. The lowest number of contaminated samples per year was observed for strawberries in Spanish situation, mainly due to the lower consumption frequency and the higher die-off of the pathogens. The highest number of contaminated samples per year was observed for basil, mainly due to the higher prevalence

(especially of *Salmonella*) in this commodity (both in the Spanish and in the Belgian situation as the same prevalence was assumed in both countries). From Table 21, it can be derived that if a Belgian consumer consumes three basil portions per year, then 0.05 portions are estimated to be contaminated with *Salmonella* (or: once in 20 years, one portion is estimated to be contaminated with *Salmonella* when consuming three portions a year), whereas daily consuming of a portion of basil can lead to five to nine contaminated portions a year.

By taking the actual prevalence into account, the positive fractions decreased, e.g. from 81.52% to 1.7%, from 60.62% to 0.2% and from 76.45% to 0.3% in case of basil, strawberries and lettuce, respectively, contaminated with *Salmonella* in the Belgian situation and from 82.44% to 1.7%, from 43.21% to 0.2% and from 66.75% to 0.2% in case of basil, strawberries and lettuce, respectively, contaminated with *Salmonella* in the Spanish situation.

Table 21: Distribution (5%, 25%, 50%, 95% and 99% percentile) of frequency of consumption in one year and the correlated portions contaminated with *Salmonella* or STEC without or with (p) taking the prevalence of *Salmonella* and STEC into account (n = 3; 100,000 iterations; average and standard deviation between three different simulations is given). Only performed in case immersion is used as wash practice.

	Basil					Strawberries					Butterhead lettuce				
		Salmonella		STEC			*Salmonella*		STEC			*Salmonella*		STEC	
Percentile	Consumption frequency	Contaminated	Contaminated (p)	Contaminated	Contaminated (p)	Consumption frequency	Contaminated	Contaminated (p)	Contaminated	Contaminated (p)	Consumption frequency	Contaminated	Contaminated (p)	Contaminated	Contaminated (p)
BELGIUM															
5%	3	2.45 ± 0.00	0.05 ± 0.00	2.45 ± 0.00	0.03 ± 0.00	1.5	0.91 ± 0.00	0.0008 ± 0.00	1.01 ± 0.00	0.0009 ± 0.00	3	2.30 ± 0.00	0.002 ± 0.00	2.30 ± 0.00	0.002 ± 0.00
25%	12	9.78 ± 0.00	0.15 ± 0.00	9.78 ± 0.00	0.09 ± 0.00	13	7.88 ± 0.00	0.01 ± 0.00	8.77 ± 0.00	0.007 ± 0.00	12	9.17 ± 0.00	0.01 ± 0.00	9.18 ± 0.00	0.01 ± 0.00
50%	13	10.60 ± 0.00	0.33 ± 0.00	10.60 ± 0.00	0.22 ± 0.00	13	7.88 ± 0.00	0.02 ± 0.00	8.77 ± 0.00	0.02 ± 0.00	39	29.81 ± 0.00	0.04 ± 0.00	29.82 ± 0.00	0.04 ± 0.00
95%	365	297.54 ± 0.00	4.64 ± 0.02	297.50 ± 0.00	3.12 ± 0.01	91.25	55.31 ± 0.00	0.19 ± 0.00	61.59 ± 0.00	0.21 ± 0.00	156	119.25 ± 0.00	0.57 ± 0.00	119.28 ± 0.00	0.57 ± 0.01
99%	365	297.54 ± 0.00	8.68 ± 0.08	297.50 ± 0.00	6.13 ± 0.00	134.42 ± 37.38	94.56 ± 0.00	0.48 ± 0.00	105.29 ± 0.00	0.54 ± 0.02	365	279.03 ± 0.00	1.43 ± 0.02	279.09 ± 0.00	1.42 ± 0.02
SPAIN															
5%	1.5	1.24 ± 0.00	0.03 ± 0.00	1.24 ± 0.00	0.02 ± 0.00	1.5	0.65 ± 0.00	0.0002 ± 0.00	0.73 ± 0.00	0.0003 ± 0.00	12	8.01 ± 0.00	0.008 ± 0.00	8.02 ± 0.00	0.008 ± 0.00
25%	12	9.89 ± 0.00	0.19 ± 0.00	9.89 ± 0.00	0.12 ± 0.00	1.5	0.65 ± 0.00	0.001 ± 0.00	0.73 ± 0.00	0.002 ± 0.00	156	104.13 ± 0.00	0.07 ± 0.00	140.22 ± 0.00	0.07 ± 0.00
50%	52	42.87 ± 0.00	0.85 ± 0.00	42.88 ± 0.00	0.54 ± 0.00	3	1.30 ± 0.00	0.004 ± 0.00	1.46 ± 0.00	0.005 ± 0.00	156	104.13 ± 0.00	0.24 ± 0.00	104.22 ± 0.00	0.24 ± 0.00
95%	365	300.89 ± 0.00	5.36 ± 0.02	300.96 ± 0.00	3.72 ± 0.02	39	16.85 ± 0.00	0.06 ± 0.00	18.96 ± 0.00	0.07 ± 0.00	365	243.64 ± 0.00	1.86 ± 0.01	243.85 ± 0.00	1.87 ± 0.01
99%	1095	902.66 ± 0.00	12.33 ± 0.08	902.88 ± 0.00	8.35 ± 0.10	91.25	39.43 ± 0.00	0.18 ± 0.00	44.36 ± 0.00	0.20 ± 0.00	1095	730.91 ± 0.00	4.02 ± 0.05	731.54 ± 0.00	4.00 ± 0.07

6.3.2 Scenario and sensitivity analysis

In the first scenario it was assumed that all consumers stored the considered fresh produce commodities in the refrigeration, while in the second scenario the effect of washing the commodities on the distribution of number of portions contaminated per year (taking the prevalence into account) was determined. Results are summarized in Table 22.

By storing all the commodities in the refrigerator a reduction of the spoiled fraction was observed. Only for basil consumed in the Belgian situation, an increase in positive fraction was observed, due to the biggest decrease in spoilage (as from the consumer survey it appeared that only 37% of consumers store fresh-cut basil in refrigerator). However, a limited effect was observed on the number of portions contaminated. Changing the washing practices (all consumers or none of the consumers wash the commodities) has no influence on the spoiled fraction. When washing all commodities, the largest decrease in positive fraction was observed in case of basil as only 68% (Belgian situation) and 77% (Spanish situation) wash basil. However, similar numbers of contaminated samples were found compared to the basic scenario, indicating the limited effect if all consumers would wash the commodities. When no washing of the commodities was applied by the consumers an increase of at least 10% in positive fraction was observed. Subsequently, the number of contaminated portions was 1.17 to 1.28 times higher compared to the basic scenario for the 99% percentile (P99). For example, if 365 portions (P99) a year are consumed in the Belgian situation, almost nine portions of the 365 consumed may be contaminated with *Salmonella* per year (basic scenario). If none of the consumers wash basil than ten portions may be contaminated with *Salmonella*.

Table 22: Results of scenario 1 and 2 being the positive and spoiled fractions for each scenario and distribution (P50, P95 and P99 describing the 50%, 95% and 99% percentile respectively) of number of contaminated portions per year in the Belgian and Spanish situation (taking the prevalence of *Salmonella* and STEC into account). Calculation were only performed for the wash practice 'immersion' (n = 3; 100,000 iterations; B = basil, S = strawberries, L = lettuce). Bold values indicate that the number of portions is more than 1.1 times (10%) higher compared to the basic scenario (only performed for P99).

			Basic scenario				Scenario 1[a]					Scenario 2a[b]					Scenario 2b[c]				
			Positive (%)	Spoiled (%)	P95	P99	Positive (%)	Spoiled (%)	P50	P95	P99	Positive (%)	Spoiled (%)	P50	P95	P99	Positive (%)	Spoiled (%)	P50	P95	P99
Belgium	*Salmonella*	B	81.52 ± 0.11	4.44 ± 0.03	4.64 ± 0.02	8.68 ± 0.08	85.31 ± 0.04	0.00 ± 0.00	0.34 ± 0.00	4.88 ± 0.03	9.06 ± 0.15	74.82 ± 0.04	4.39 ± 0.01	0.30 ± 0.00	4.26 ± 0.02	**7.92 ± 0.06**	95.57 ± 0.03	4.43 ± 0.03	0.38 ± 0.00	5.46 ± 0.04	**10.15 ± 0.04**
		S	60.62 ± 0.01	1.44 ± 0.01	0.19 ± 0.00	0.48 ± 0.00	60.59 ± 0.05	0.00 ± 0.00	0.02 ± 0.00	0.19 ± 0.00	0.49 ± 0.00	58.95 ± 0.06	1.45 ± 0.02	0.02 ± 0.00	0.18 ± 0.00	0.47 ± 0.01	70.72 ± 0.09	1.44 ± 0.04	0.02 ± 0.00	0.22 ± 0.00	**0.58 ± 0.01**
		L	76.45 ± 0.09	2.69 ± 0.01	0.57 ± 0.00	1.43 ± 0.02	77.17 ± 0.04	1.91 ± 0.00	0.04 ± 0.00	0.57 ± 0.00	1.43 ± 0.01	76.10 ± 0.05	2.70 ± 0.04	0.04 ± 0.00	0.56 ± 0.01	1.40 ± 0.02	97.31 ± 0.01	2.69 ± 0.01	0.06 ± 0.00	0.72 ± 0.00	**1.83 ± 0.03**
	STEC	B	81.51 ± 0.10	4.44 ± 0.03	3.12 ± 0.01	6.13 ± 0.00	85.26 ± 0.09	0.00 ± 0.00	0.23 ± 0.00	3.24 ± 0.01	6.39 ± 0.08	74.83 ± 0.03	4.38 ± 0.01	0.20 ± 0.00	2.87 ± 0.02	5.64 ± 0.08	95.62 ± 0.03	4.38 ± 0.03	0.26 ± 0.00	3.66 ± 0.02	**7.16 ± 0.02**
		S	67.49 ± 0.15	1.44 ± 0.01	0.21 ± 0.00	0.54 ± 0.02	64.68 ± 0.05	0.00 ± 0.00	0.02 ± 0.00	0.20 ± 0.00	0.52 ± 0.00	65.77 ± 0.07	1.41 ± 0.06	0.02 ± 0.00	0.21 ± 0.00	0.52 ± 0.01	78.57 ± 0.10	1.43 ± 0.04	0.03 ± 0.00	0.25 ± 0.00	**0.63 ± 0.01**
		L	76.46 ± 0.12	2.69 ± 0.01	0.57 ± 0.01	1.42 ± 0.02	77.12 ± 0.05	1.91 ± 0.00	0.04 ± 0.00	0.57 ± 0.00	1.45 ± 0.01	76.08 ± 0.07	2.71 ± 0.04	0.04 ± 0.00	0.57 ± 0.01	1.42 ± 0.00	97.31 ± 0.01	2.69 ± 0.01	0.06 ± 0.00	0.73 ± 0.00	**1.71 ± 0.01**
Spain	*Salmonella*	B	82.44 ± 0.09	0.83 ± 0.03	5.36 ± 0.02	12.33 ± 0.08	83.22 ± 0.02	0.00 ± 0.00	0.86 ± 0.00	5.39 ± 0.01	12.48 ± 0.19	77.53 ± 0.21	0.79 ± 0.03	0.80 ± 0.00	5.03 ± 0.01	11.58 ± 0.02	99.15 ± 0.03	0.85 ± 0.03	1.03 ± 0.00	6.42 ± 0.03	**14.68 ± 0.09**
		S	43.21 ± 0.09	0.86 ± 0.02	0.06 ± 0.00	0.18 ± 0.00	43.14 ± 0.10	0.00 ± 0.00	0.004 ± 0.00	0.06 ± 0.00	0.18 ± 0.00	42.75 ± 0.07	0.88 ± 0.00	0.004 ± 0.00	0.06 ± 0.00	0.18 ± 0.00	51.75 ± 0.08	0.87 ± 0.04	0.005 ± 0.00	0.07 ± 0.00	**0.21 ± 0.00**
		L	66.75 ± 0.08	14.89 ± 0.02	1.86 ± 0.01	4.02 ± 0.05	67.13 ± 0.07	14.51 ± 0.00	0.24 ± 0.00	1.88 ± 0.01	4.02 ± 0.02	66.59 ± 0.04	14.91 ± 0.01	0.24 ± 0.00	1.87 ± 0.02	3.97 ± 0.07	85.11 ± 0.01	14.89 ± 0.01	0.31 ± 0.00	2.38 ± 0.01	**5.04 ± 0.08**
	STEC	B	82.45 ± 0.07	0.83 ± 0.03	3.72 ± 0.02	8.35 ± 0.10	83.15 ± 0.05	0.00 ± 0.00	0.54 ± 0.00	3.76 ± 0.01	8.53 ± 0.06	77.59 ± 0.03	0.83 ± 0.04	0.51 ± 0.00	3.51 ± 0.03	7.88 ± 0.21	99.20 ± 0.03	0.80 ± 0.03	0.65 ± 0.00	4.48 ± 0.01	**10.18 ± 0.15**
		S	48.61 ± 0.03	0.86 ± 0.02	0.07 ± 0.00	0.20 ± 0.00	47.99 ± 0.11	0.00 ± 0.00	0.005 ± 0.00	0.07 ± 0.00	0.20 ± 0.00	48.33 ± 0.07	0.87 ± 0.02	0.005 ± 0.00	0.07 ± 0.00	0.20 ± 0.00	58.67 ± 0.11	0.87 ± 0.03	0.006 ± 0.00	0.08 ± 0.00	**0.24 ± 0.00**
		L	66.81 ± 0.11	14.89 ± 0.02	1.87 ± 0.01	4.00 ± 0.07	67.09 ± 0.09	14.51 ± 0.00	0.24 ± 0.00	1.88 ± 0.01	3.99 ± 0.03	66.53 ± 0.06	14.90 ± 0.01	0.24 ± 0.00	1.87 ± 0.01	4.00 ± 0.01	85.10 ± 0.01	14.90 ± 0.01	0.31 ± 0.00	2.38 ± 0.02	**5.04 ± 0.05**

[a]: 100% refrigeration, [b]: All consumers wash the commodity before consumption, [c]: None of the consumers wash the commodity before consumption

In the third scenario, the influence of the country of origin of basil (and thus the prevalence of *Salmonella* on basil) on the number of contaminated portions was determined. STEC was not taken into account as the prevalence was very low (only based on PCR results, no STEC isolates obtained) and similar in all countries (CHAPTER 4). From Table 23, it can be derived that if basil is cultivated in Cyprus, the number of samples contaminated with *Salmonella* is 3.25 to 5.53 times higher compared to basil cultivated in Belgium and consumed as in the Belgian situation. In general, the lowest number was found for basil cultivated in Belgium, followed by cultivation in Israel and Cyprus, because the detected prevalence was respectively 0/100 for basil originating from Belgium, 2/100 from Israel and 3/92 from Cyprus.

In the last scenario 4, the influence of the definition for the presence of pathogenic *E. coli* on the number of contaminated portions was determined. STEC was defined as the presence of *stx* and *eae* genes in the samples (detection by PCR), while $STEC^+$ was defined as the presence of a serotype and the most likely associated *eae*-variant in addition to the *stx* gene (detection by PCR) (CHAPTER 4). It should be noted that no pathogenic *E. coli* was isolated. Using the conservative STEC definition resulted in 3 to 5 or 7 times more contaminated samples compared to the $STEC^+$ definition.

Table 23: Overview of the ratio (e.g. number of contaminated samples in Israel divided by number of contaminated samples in Belgium) for scenario 3 and 4. P50, P95 and P99 are describing the 50%, 95% and 99% percentile respectively. Calculation were only performed for basil and the wash practice 'immersion' (n = 3; 100,000 iterations).

		Scenario 3 (*Salmonella*)			Scenario 4 (STEC)
		Israel vs. Belgium	Cyprus vs. Belgium	Cyprus vs. Israel	STEC vs. $STEC^+$
Belgium	**P50**	3.77	5.53	1.47	5.13
	P95	2.90	4.12	1.42	3.83
	P99	2.40	3.25	1.36	3.01
Spain	**P50**	4.78	7.35	1.54	6.80
	P95	2.52	3.51	1.39	3.24
	P99	2.48	3.49	1.41	3.17

Secondly, a tornado plot was developed to determine the influence of the parameters "initial concentration" and "removal by washing" on the output results (= number of contaminated portions taking the prevalence into account) (Figure 27). At the 99%

percentile the largest variation can be observed. In addition, a larger variation was observed for the parameter initial concentration (0.10 cfu/g versus 100 cfu/g) compared to removal by washing (25.11 cfu/g versus 1.20 cfu/g).

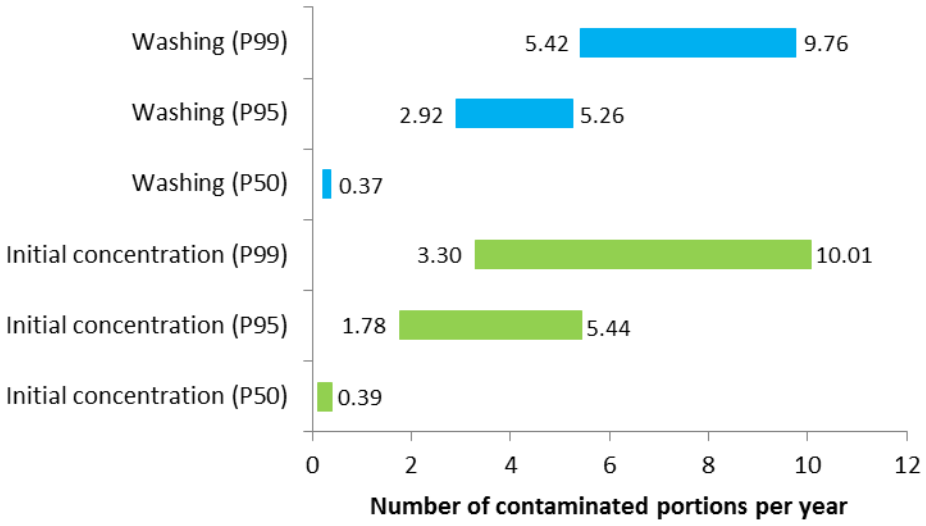

Figure 27: Influence of inserting the minimum and maximum of the distribution (as inserted in the basic scenario) of parameters 'initial concentration' and 'removal by washing', on the number of contaminated basil portions consumed in the Belgian situation for the 50% (P50), 95% (P95) and 99% (P99) percentile (n = 3; 100,000 iterations).

6.4 Discussion

A quantitative microbiological risk assessment (QMRA) describes the propagation of a pathogen from farm to retail and consumer's home with a potential estimation of the number of human cases of illness and is thus capable of estimating the public health effect of control measures issued for a specific pathogen-food product combination. However, due to the extensive range of fresh produce commodities (and often several relevant hazards which are associated with a single commodity) along with many uncertainties, variability and insufficient characterization and knowledge of interaction of the many factors along the farm to fork supply chain impacting the behavior of foodborne pathogens on fresh produce, a prioritizing manner is often preferred (FAO/WHO, 2008). Risk ranking can be used to identify and subsequently prioritize the most significant commodity, in combination with a specific pathogen, for a given situation. A qualitative ranking using a set of criteria was

performed by the FAO and WHO to order the fresh produce of concern. Leafy green vegetables were ranked as the highest priority, followed by berries, sprouted seeds, etc. in terms of fresh produce (FAO/WHO, 2008). In the FDA ranking tool for fresh produce (Anderson et al., 2011), scores were added to eight criteria (Table 4) and an overall rank per pathogen-commodity pair was calculated by developing an algorithm that balances the score for each criterion. Leafy greens (such as lettuce and spinach) in combination with *E. coli* O157:H7 were found to be the top ranked commodity. In the EFSA risk ranking of foods of non-animal origin (FoNAO), based on modification of the model by Anderson et al. (2011), leafy greens eaten raw in combination with *Salmonella* was identified as the highest priority (EFSA, 2013b). In both cases this can be explained by, among others, the high number of outbreaks identified with leafy greens and the severity of the illness caused by *E. coli* O157:H7 and *Salmonella*. Fresh herbs and strawberries in combination with *Salmonella* or STEC, were not observed in the top five ranked groups neither in the US FDA study nor in the EFSA study based on qualitative ranking (Anderson et al., 2011; EFSA, 2013b).

In the present study, risk characterization, outbreak association and severity of the disease were not taken into account, in contrast to other risk ranking studies described in Table 4 (CHAPTER 1). On the other hand, the survival of *Salmonella* and *E. coli* O157:H7 in relation to consumer handling and the measured prevalence (in Belgium and Norway) was considered. Instead of addressing a score, the influence of several parameter on the presence of *Salmonella* or STEC on basil, strawberries and (butterhead) lettuce was quantitatively determined.

The highest risk in our study (top priority) was attributed to basil in association with *Salmonella*, irrespective of the variability of consumer behavior or consumption patterns within Europe. In addition, using even more detailed prevalence data, a difference in relative risk for basil was noted depending upon the country of origin (with highest risk originating from Cyprus, lowest if originating from Belgium). Similar survival of *Salmonella* and STEC on basil and lettuce during storage was observed, but the prevalence on lettuce was lower than on basil which affects the final output. However, even by assuming that all basil was cultivated in Belgium, the highest priority was found for basil due to different consumers handling compared to lettuce (e.g. less washing of basil before consumption). Furthermore, it was observed that changing storage and washing practices by consumers in the model has

only a limited effect on the final output (see scenario analysis). The lowest risk was attributed to strawberries mainly due to the lower consumption frequency but also the higher die-off of the enteric pathogens on strawberries versus basil leaves or lettuce during storage.

In the present study, basil, as niche product, was found to be as important or even more important than lettuce. Moreover, both commodities have already been associated with foodborne outbreaks of *Salmonella* and pathogenic *E. coli* (STEC and ETEC) in Europe (Horby et al., 2003; Nygard et al., 2008; Pakalniskiene et al., 2009; Pezzoli et al., 2008; Soderstrom et al., 2008; Takkinen et al., 2005). This indicates the importance of including fresh herbs (and leafy greens) in monitoring and surveillance surveys. In addition, the country of origin appeared to be important with regard to the prevalence, which highlights the need of increased attention for good practices, and should be included in the monitoring and surveillance. On the other hand, strawberries were regarded as quite safe, as they were found to have to lowest risk and no foodborne outbreaks associated with *Salmonella* or STEC were yet reported in Europe. Therefore, no increased attention on strawberries, with regard to enteric pathogens, is needed and the production practices as in place are performing well and should be maintained.

From the scenario analysis it was noted that the risk ranking is a combination of many factors but the driving force is mainly the prevalence of pathogens. But not only the prevalence will have a major impact on the outcome of the exposure assessment model. It is expected that also the (assumed) concentration of pathogens in the positive contaminated samples will have an impact. The initial concentration of *Salmonella* or STEC on contaminated fresh vegetables and fruits is assumed to be low. In Pielaat et al. (2008), the initial concentration of *Salmonella* and *E. coli* O157:H7 was determined on contaminated raw produce (endive, iceberg lettuce and Oak three lettuce). The *Salmonella* counts were found in the range of 0.019 cfu/g to more than 0.281 cfu/g, whereas *E. coli* O157:H7 was estimated at 0.052 cfu/g by using the MPN method. In Danyluk & Schaffner (2011), a start level of 1 cfu/10 g was used for the quantitative assessment of microbial risks on leafy greens. It should also be noted that the contamination is not evenly distributed over the commodity (as mostly point contamination occurs, e.g. contact with soil or manure (Heaton & Jones, 2008)). During sensitivity analysis, it was observed that, when consuming daily a

portion of basil, the number of portions contaminated was 3.06 times higher when the maximum value (100 cfu/g) of initial concentration was inserted instead of the minimum value (1 cfu/10 g). Washing fresh vegetables and fruit with water can reduce the number of bacteria, but not eliminate them (Hung et al., 2010; Pangloli et al., 2009). It is assumed that bacteria can hide in plant structures (e.g. stomata or achenes), avoiding removal (Koseki et al., 2004; Koseki et al., 2003). However, the exposure assessment model may estimate the removal of all pathogenic cells by washing at consumer's home, when only a low number of cells is present after storage, possibly resulting in a underestimation of the positive fraction derived from the model.

Moreover, it should be noted that the number of samples, analyzed during the screening of the commodity for pathogens, will impact on the estimated prevalence and thus also have an influence on the output of the model. The prevalence was inserted as an Beta-distribution to account the uncertainty in the prevalence. Even though no pathogens were detected on strawberries (0/257) and on butterhead lettuce (0/279), the average prevalence was 0.386% and 0.356%, respectively. Moreover, the higher the number of samples analyzed (and negative for pathogens), the lower the average prevalence (e.g. 0/2570 → average prevalence = 0.0389 %). Overall, both *Salmonella* and STEC are widely acknowledged as pathogens of major importance and in the framework of this risk ranking exercise it was not considered the prior objective to calculate the risk actually based on the number of illnesses. A ranking based on an exposure assessment model was preferred instead. Thus, in the present study, the number of contaminated portions was used to rank pathogen-commodity combinations for their importance to pose a risk to the consumers.

In conclusion, this risk ranking calculation demonstrates that also niche products such as basil leaves can have an impact on public health equal or higher than lettuce whereas strawberries indeed are confirmed to be quite safe fruit items. In the ranking, the prevalence of *Salmonella* and STEC on the selected fresh produce items was the main driving factor. A moderate effect on the ranking was found for the consumption frequency, while the consumer behavior (such as time/temperature of storage or washing practices) influences the ranking to a minor extent. This stresses the need for priority in food safety protection on monitoring and surveillance and thus to collect further representative data on presence of these enteric pathogens on all types of fresh herbs (such as basil) as well as other leafy

greens used in salads. Furthermore, it indicates continuous and increased attention to good agricultural or manufacturing practices in all types of commodities is needed. Efforts to reduce the risk by education campaigns on safe handling practices in the kitchen are known to be quite challenging in getting the message through and seem also to be only having a minor effect on the relative risk that consumers will be exposed.

CHAPTER 7: General discussion

7.1 Enteric pathogens on fresh produce

7.1.1 Prevalence of enteric pathogens on fresh herbs and strawberries

The microbiological safety of fresh fruit and vegetables has become a concern worldwide, due to the increased association with foodborne outbreaks. Moreover, ready-to-eat (RTE) fresh fruit and vegetables are mostly consumed raw without any intervention step (e.g. heating) to kill harmful microorganisms. Fresh fruit and vegetables at harvest have a natural epiphytic microbiota. Most of these microorganisms are non-pathogenic, but microbial contamination may occur during any step from cultivation to consumers from a variety of sources and this may include pathogens (bacteria, parasites or viruses) (FAO/WHO, 2008). The prevalence of *Salmonella* and STEC on fresh produce is considered low. However, depending on the origin of the commodity, a higher prevalence may be found. In Table 24, some examples of surveys performed in Europe are listed. Due to the higher association of fresh herbs with RASFF notifications and foodborne outbreaks, more investigations on the microbiological safety were performed for these commodities, in particular more surveys compared to strawberries which are overall recognized as safe. However, information about the microbiological safety of fresh herbs cultivated in non-Asian countries is rather limited. In the present PhD study, the microbiological safety of fresh herbs (basil and coriander) and strawberries was assessed. No *Salmonella* or STEC were detected on strawberry fruits itself. In case of fresh herbs, *Salmonella* could be isolated from basil and coriander which were sourced from Israel (1.0%) and from Cyprus (4.2%). However, no illness relating to fresh herbs such as basil and coriander, has been reported yet in Belgium. On the other hand, illness related to basil associated with *Salmonella* has been reported in the UK (Pezzoli et al., 2008) and Denmark (Pakalniskiene et al., 2009), for which basil was imported from Israel in both cases. A possible explanation is related to the infectivity of certain *Salmonella* strains. It is reported that almost all *Salmonella* serotypes can cause disease, but a wide variation in response (probability of infection) was observed between species of *Salmonella* and even from strain to strain of the same species (Teunis et al., 1996). The major subspecies responsible for foodborne outbreaks of *Salmonella* with regard to fresh produce, is *enterica*. In 6 out of 10 contaminated herb samples in the PhD study, subspecies *salamae* or *diarizonae* were detected. Both subspecies have been isolated from dried spices (mint and anise), but were not yet related to foodborne outbreaks of fresh produce (Van Doren et al.,

2013). This may suggest that high levels, which are normally not expected to be present on food products, are needed to cause disease for these strains. Moreover, the usual habitat for these subspecies are cold-blooded animals (e.g. reptiles) and the environment, in contrast to the subspecies *enterica* which usual habitat are warm-blooded animals (Brenner et al., 2000). Another possibility is the underreporting of illnesses related to *Salmonella* which depends on the probability that a person with diarrhea visits a physician, a stool is taken and tested for pathogens, the sensitivity of the laboratory test and the notification of *Salmonella* positive stool samples to the national surveillance system. The under-ascertainment depends on the severity of the illness (Gibbons et al., 2014; Hall et al., 2008). Data from the Netherlands suggest for *Salmonella* the use of a disease multiplier of 57.5 to estimate the actual number of cases from the number of reported cases (Havelaar et al., 2013).

Table 24: A selection of prevalence studies of *Salmonella* and *E. coli* O157 on fresh (cut) fruit and vegetables (from retail) in Europe

	Prevalence *Salmonella*	**Prevalence of *E. coli* O157**	**Country**	**Origin**	**Reference**
Fresh herbs (e.g. basil, chives, coriander, etc.)	18/3760 (0.5%)	-	UK	Israel, Spain or NK[a]	(Elviss et al., 2009)
Fresh herbs (basil, coriander, etc.)	32/244 (13.1%)[c]	-	UK	Non-EU countries	(Surman-Lee et al., 2008)
	5/298[d] (1.7%)	-	UK	India, Thailand or NK[a]	
Fresh (cut) fruit and vegetables	4/300 (1.3%)	-	Spain	NK[a]	(Abadias et al., 2008)
Strawberries	0/173	0/173	Norway	Norway or imported[b]	(Johannessen et al., 2002)
Growing herbs (pots)	0/130	0/130	Norway	Norway	
Lettuce	0/138	0/138	Norway	Norway or imported[b]	
Pre-packed parsley and dill	0/38	/038	Norway	Norway or imported[b]	
Fresh herbs and leafy vegetables (mint, basil, coriander, etc.)	24/159[f] (15.1%)	-	Norway	East Asia[e]	(NSCFS, 2008)
	45/162[d] (27.8%)	-	Norway		
Fresh vegetables (endive, cucumber, iceberg lettuce, etc.)	1/1151 (0.1%)	0/1151	Netherlands	NK[a]	(Wijnands et al., 2014)

[a]: not known due to lack of available data, [b]: countries not specified, [c]: survey at Border Inspection Post in 2005, [d]: survey performed in 2007, [e]:mainly Thailand and Vietnam, [f]: survey performed in 2005

No STEC was isolated from fresh basil or coriander. In the present PhD study, the prevalence of STEC was assessed, instead of focusing on *E. coli* O157 as found in most prevalence studies or monitoring and surveillance surveys (EFSA, 2014a). As non-O157 *E. coli* are increasing in importance regarding to foodborne outbreaks, investigation/monitoring of STEC O157 alone may lead to an underestimation of the prevalence of STEC on food.

Microbiological testing is frequently used to determine the microbiological quality and safety of foods. The low prevalence of enteric pathogens on fresh produce makes it highly unlikely to find positive results in case only a restricted number of samples can be taken. In the present PhD study, enteric pathogens were only detected on herbs during a few weeks within a whole year of sampling highlighting the need for frequent and year-round sampling in monitoring for pathogens' contamination. But, the latter is time consuming and expensive for a company or competent authority which have to deal with limitation in resources. Moreover, homogenous pathogen contamination of individual units within a batch of fresh produce is also not expected. Zwietering et al. (2014) showed that for pathogens the performance of sampling is rather poor even when high numbers of samples are tested, if the sampling is required to be able to detect a low rate of contaminated products. It was observed that even a sampling plan of 60 samples per batch has a quite low probability of detecting contamination rates of 1% or 2% as the probability of acceptance is still 55% and 30% respectively.

7.1.2 Contamination level of enteric pathogens on fresh produce.

From present PhD study and other surveys, it is noted that fresh produce put on the market in Belgium (or Europe) most likely has a low (0.1-1%) contamination rate of pathogens. In addition, there is limited information available in the literature concerning the level of contamination of fresh produce (the numbers of *Salmonella* and STEC present per gram in case of a positive sample). This because the detection of *Salmonella* and STEC is relying on presence/absence testing of the pathogen in 25 g and usually no enumerations are performed. (Pielaat et al., 2008) estimated the initial concentration of *Salmonella* and *E. coli* O157:H7 present on contaminated raw produce (endive, iceberg lettuce and Oak three lettuce) using the MPN method and the estimated numbers were very low: 0.019 cfu/g to more than 0.281 cfu/g for *Salmonella* and 0.052 cfu/g for *E. coli* O157:H7. To deal with these

low contamination levels, detection methods include an enrichment step to enable growth of the target pathogen. Moreover, enrichment serves for resuscitation of injured or stressed target cells and dilution of food inhibitors and background flora (Wang et al., 2013).

Fresh produce is characterized by high levels of background microbiota. The number and type of microorganisms present depends on the fresh produce commodity, but also on the cultivation type, environment, the season, etc. (Lindow & Brandl, 2003). Using a selective enrichment may favor the growth of the target pathogen while suppressing the growth of non-target background bacteria. However, the use of a selective enrichment broth may suppress the growth of stressed or injured target cells (e.g. due to the harsh environmental conditions such dried stress and UV irradiation) (Jasson et al., 2009). Non-selective enrichment, such as Buffered Peptone Water (BPW), can be used, but care should be taken that target bacteria are not overgrown by non-target microorganism which are most likely present in higher numbers on fresh produce (Tzschoppe et al., 2012). In the present study, the numbers for coliform bacteria on basil and coriander ranged from 1.6 to 7.5 log cfu/g. Moreover, the preparation of the sample before enrichment may have an influence on the enrichment or disturb detection. By mechanical homogenization (stomaching) compounds of the food item are released in the broth and may inhibit bacterial growth during enrichment or the subsequent PCR reaction (Jacobson et al., 2012; Wilson, 1997).

Therefore, it is important to evaluate the detection method for their application in specific cases. In the present study, a non-selective enrichment media BPW was preferred for the detection of *Salmonella* and STEC on fresh herbs and strawberries by PCR, whereby good results were obtained. To isolate these pathogens from the commodities, a selective enrichment was followed in the case of *Salmonella*, to deal with the background microbiota. On the other hand, no selective enrichment in mTSB+N (developed for the isolation of STEC O157) was applied for the isolation of STEC, as growth of some non-O157 STEC in enrichment broth with novobiocin was inhibited (Verstraete et al., 2012; Vimont et al., 2007). After non-selective enrichment, IMS can be applied to concentrate the target bacteria. However, immunomagnetic beads are only available for most common serotypes of STEC (O157, O26, O111, O103, O145, etc.), whereby uncommon STEC serotypes, which also may cause severe illness, would be missed. In addition, testing samples for all these serotypes by IMS would be labor intensive and time consuming. After evaluating the

detection method applied in the present study, troubled reading of selective agars and inhibition during PCR reaction was mainly observed for basil, possibly due to the presence of high levels of background microbiota and phenolic compounds, respectively (Jayasinghe et al., 2003). During the present study, coliform bacteria were isolated from basil leaves during storage at different temperatures by plating on VRBL-agar. Therefore, individual basil packages from the same batch were used. Subsequently, five colonies were picked from the agar plated and identified by MALDI-TOF (Figure 28). It was observed that *Enterobacter cloacae* was the most frequently isolated strain on the investigated batch. In addition, more different genera were found on basil leaves during storage at 7°C and 15°C compared to storage at 22°C. Probably, *Enterobacter* overgrew the other genera at 22°C.

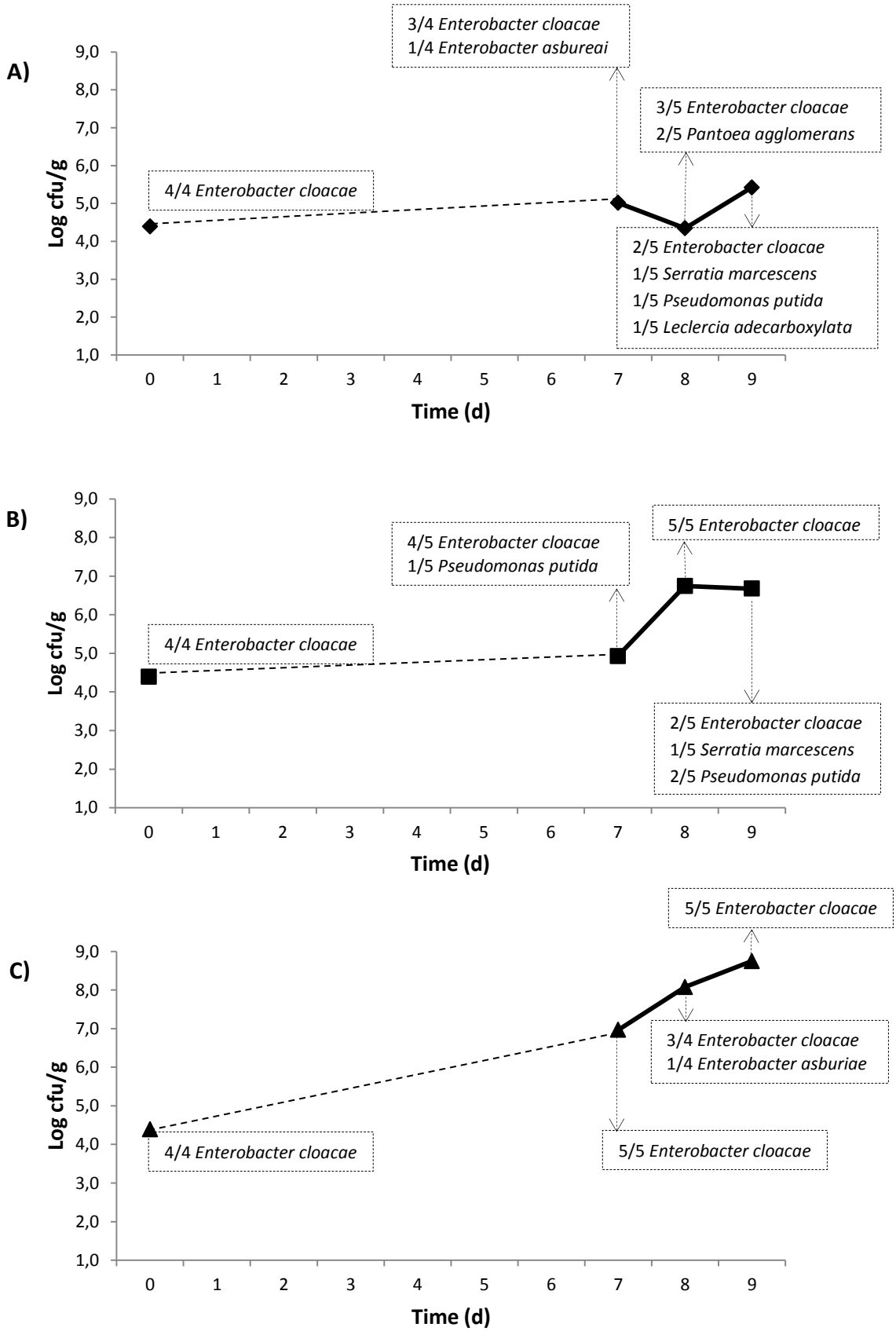

Figure 28: Evolution of coliform bacteria (plated on VRBL-agar) on basil leaves during storage at 7°C (A), 15°C (B) and 22°C (C). Five colonies were picked from the VRBL-agar and identified by MALDI-TOF (1 missing sample on day 0)

7.1.3 Detection of STEC on fresh produce

Rapid microbiological detection methods are needed to rapidly diagnose and treat patients and to enable the prompt notification of outbreaks and implementation of control measures to prevent more cases. Sensitive methods are needed, as these pathogens may only be present in food and environmental samples in small numbers, to identify the reservoir of STEC and the routes of transmission to humans and to ensure a safe supply of foods. Moreover, sensitive methods are needed for surveillance programs in risk assessment studies and for studies on the survival and growth of STEC strains. The recovery of low numbers of STEC during the enrichment procedures depends on the competing flora present, both the total number of bacteria and more especially, the number of Enterobacteriaceae. The possible presence of different serotypes of STEC (more than 100 serotypes have been isolated from patients with diarrhea or HUS), non-toxigenic strains of common STEC serotypes associated with illness and generic *E. coli* makes the detection of STEC strains even more complex. For example, isolation is hampered because among others non-O157 STEC strains resemble generic *E. coli,* different substrates are fermented by different serotypes and the sensitivity to selective component, used for the differentiation with background microbiota, differ between serotypes (De Boer & Heuvelink, 2000).

EFSA (2013c) states that there is no single or combination of marker(s) that defines a pathogenic STEC. However, RTE products contaminated with an isolate of one of the STEC serotypes (O157, O26, O103, O145, O111 and O104) in combination with *stx* and *eae* or *aaiC* and *aggR* genes were considered as presenting a potentially high risk for diarrhea and HUS. During the present screening, no STEC were isolated from fresh herbs and strawberries. However, in 11 herb samples *stx1* and/or *stx2* and *eae* genes were detected by PCR. Subsequently, the samples was analyzed for the presence of one of the *eae*-variants (β, γ, ε, θ) and genes for a serotype (O26, O103, O145, O111). In 6 out the 11 herb samples, a serotype could be detected and 3 of them showed the presence of the most likely associated *eae*-variant for a certain serotype (Bibbal et al., 2014; Madic et al., 2010; Oswald et al., 2000; Posse et al., 2007). Detection of *stx* and *eae* genes by PCR from the enrichment broth of a food commodity (= presumptive presence of STEC), does not necessarily imply that a viable STEC strain (presence of *stx* and *eae* gene) is present. It is possible that *stx* and *eae* genes are originating from different bacteria, that free *stx* phages were present or that *eae* originates

from other bacteria than *E. coli* (Bettelheim, 2003; Beutin et al., 2009). Including the detection of *eae*-variants and the most common serotypes associated with severe disease, increases the likelihood for the actual presence of (highly) pathogenic STEC, especially when a serotype and the most likely associated *eae*-variant and *stx* are detected (= presumptive presence of highly pathogenic STEC). But, care should be taken, as also other serotypes may cause severe illness and would be missed when focusing on PCR positive samples for *stx* and a serotype with the most likely associated *eae*-variant. Screening methods targeting virulence gene profiles of STEC irrespective of their serotypes, would be better suited, but various virulence gene profiles occur among pathogenic STEC strains and the full combination of virulence factors necessary to cause disease still remain to be defined. In contrast to culture methods, which are time-consuming, labor-intensive and often associated with a low rate of STEC recovery from food, PCR based methods are characterized by reduced analysis time and high-throughput analysis capabilities. Moreover, they offer the possibility to quickly rule out negative samples (Farrokh et al., 2013). Three herb samples were reported as presumptive presence for highly pathogenic STEC O111, O103 and/or O26, even though no isolates were obtained in the present study, which may be due to the lack of a well-developed isolation method for STEC. Especially in the case of fresh leafy greens, isolation is hampered due to the high level of Enterobacteriaceae present on these commodities.

Neither in fresh herbs and strawberries, nor in environmental samples (e.g. irrigation water, substrate, animal feces and surfaces of black plastic covering the soil in strawberry cultivation), the *aggR* gene was detected by PCR. This supports that the outbreaks associated with STEC O104:H4 in Germany in 2011, was a rare event and the presence of this *aggR* gene is, in contrast to the *eae* gene, rare in the environment.

7.1.4 Survival of *Salmonella* and STEC on basil and strawberries during storage

Both *Salmonella* and STEC could survive on basil leaves and (to a lesser extent) on strawberries, especially at the recommended storage temperatures (15°C for basil, refrigeration (< 7°C) for strawberries). It should be noted that in the present study, a high initial contamination level was used, to be able to enumerate and follow survival during

storage. This high contamination levels of *Salmonella* and STEC are not likely to be present on fresh vegetables and fruit. Nevertheless, survival of both pathogens on these commodities during storage is also expected when a lower initial contamination level is used (Manios et al., 2013). In the case of strawberries, the detection limit (= 50 cfu/g) for the enumeration of *Salmonella* and *E. coli* O157 was reached towards the end of the storage experiment. However, this does not necessarily mean that pathogens are absent on strawberries. It is possible that they are still present in very low number.

Moreover, washing fresh vegetables and fruit with water cannot ensure complete removal of bacterial pathogens from the surface of these commodities. Pathogens, such as *Salmonella* and *E. coli* O157:H7 can attach to the surface, trichomes, stomata and cut edges or even be entrapped 20 to 100 µm below the surface in stomata, crevices and cut edges. This potential localization of human pathogens in the phyllosphere renders foodborne pathogens protected from washing and thus removal (Erickson, 2012; Golberg et al., 2011; Keskinen & Annous, 2011; Seo & Frank, 1999; Yu et al., 2001). In case of strawberries, the rough surface and the presence of numerous surface-borne achenes (seeds) may provide sites for the bacteria to attach and become less accessible for removal (Yu et al., 2001).

The survival of *Salmonella* and *E. coli* O157:H7 on basil and strawberries and the inability of removing all bacterial cells by washing, demonstrates that washing as an intervention will achieve a reduction in numbers (ca. 90% removal of bacteria), but it will still not result in a complete removal for fresh and RTE produce which is consumed raw. This denotes once again the importance of preventing contamination with human pathogens during cultivation and further in the food chain.

7.2 Environmental pressures on strawberry cultivation in Belgium

Neither in soilless cultivation, nor in open field soil cultivation, enteric pathogens *Salmonella* and STEC were detected on strawberries (0/72). As strawberry samples taken at farm level were pooled per three for microbiological analysis, the result could be extended to 0/216. This result may suggest safe strawberry production in Belgium. However, depending on the farm, some pressure from the environment was noticed, especially for STEC. Several

different cultivation systems (soilless vs. soil cultivation, greenhouse vs. plastic tunnels vs. open field) and environmental factors (e.g. presence of farm animals and the use of non-protected surface water for irrigation), which may influence the microbiological safety, were observed for strawberry cultivation. A higher occurrence related to the presence of enteric pathogens was observed with the use of collected rain water in an open well for irrigation and when farm animals are in the neighborhood. STEC O26, containing *stx* and *eae* genes, was isolated from rain water collected in an open well, which was located in the neighborhood of farm animals, but not adjacent, i.e. no direct run-off was possible. However, insects, birds or rodents may also contaminate surface waters used for irrigation (Evans et al., 2006). Subsequently, STEC O26 was also detected in the peat substrate, which indicates the transfer of STEC O26 from the irrigation water to the peat. During sampling of the strawberries in that period of time, no STEC was detected on strawberries, which may be explained by the use of drip irrigation in the strawberry cultivation. Moreover, internalization of enteric pathogens, such as STEC, from the peat substrate via the root of the plant to the fruit is not likely (Miles et al., 2009). Nevertheless, care should be taken that no transfer of enteric pathogens from environment to the strawberry fruit occurs. For example, also cross-contamination from contact of peat substrate via food handler to the strawberries or from the irrigation water (or nearby animals' feces) via insects to strawberries might occur. However, no foodborne outbreaks associated with strawberries, produced in Belgium, and enteric bacteria, were reported yet in Belgium, the EU or elsewhere. However, frozen strawberries, imported from China, have been implicated with a norovirus outbreak in Germany (Bernard et al., 2014). Norovirus contamination is most likely due to lack of hygiene, which is also a possible transmission route of enteric bacteria as *Salmonella* and *E. coli* O157. This highlights the need of hygienic practices, especially hand hygiene, during strawberry cultivation and processing. In the US (Oregon), strawberries have been related to a foodborne outbreaks of *E. coli* O157:H7 due to the feces of a deer present in the field (Laidler et al., 2013). The latter indicates the importance of the environment of the production field regarding to a safe production of fresh produce.

7.3 Microbiological safety of basil and strawberries

The highest risk of exposure to enteric pathogens as *Salmonella* (and STEC), was observed for basil as a niche product, whereas the lowest risk was observed for strawberries. The main factor influencing the outcome of the exposure model is the prevalence of the pathogens on the crops. The consumer handling (storage time and temperature and washing) has an influence on the concentration of the pathogens to some extent. But, as both pathogens on basil and strawberries could still be detected at the end of the storage experiment and removal of enteric pathogens from these commodities by washing is not sufficient to eliminate enteric pathogens, these factors may influence the outcome less than the initial pathogen prevalence. Because *Salmonella* and STEC can be highly infectious, a drawback in the exposure model is that 100% removal of *Salmonella* and STEC from the commodities was possible in the calculations made. However, as discussed above, the effect of washing to fully eliminate the pathogen is doubtful and there might be (low numbers of) residual cells maintained on the product, also after washing. This may have an influence on the number of exposures, but to a less extent on the ranking of the commodity-pathogen combination, as the prevalence and consumption frequency (and survival of the pathogen on the commodity) have the most influence. Basil has been associated with outbreaks of *Salmonella* and Enterotoxigenic *E. coli* in different countries in Europe, while strawberries were not yet reported as cause of a foodborne outbreaks related to *Salmonella* and *E. coli* in Europe (Pakalniskiene et al., 2009; Pezzoli et al., 2008). The difference in prevalence of pathogenic bacteria on commodities may have several reasons, e.g. (i) different cultivation: soilless and soil cultivation of strawberries vs. mainly soil cultivation of basil, drip irrigation for strawberries vs. overhead irrigation of basil) or (ii) different sourcing countries of both.

The Chapter 6 in this PhD thesis aimed at ranking the risks potentially associated with predefined microbial hazards of importance in fresh produce (*Salmonella* and STEC) in predefined commodities of interest (basil as a niche product but increasingly used in culinary preparations; strawberries as a product with high economic value and lettuce, recognized to be of the highest priority in safety of fresh produce). Risk determinants relating to home storage (survival), handling (effect of washing) and consumption were identified and data was collected either by lab experiments or from literature study or consumer surveys. For

each of the 3 fresh produce commodities, the data were used in a quantitative exposure assessment and resulted in the risk of exposure to either *Salmonella* or STEC when consuming basil, strawberries or lettuce. Due to uncertainties, e.g. initial concentration of *Salmonella* or STEC on contaminated fresh produce and subsequent removal during washing, exact calculation of the risk for human disease upon exposure is not feasible. However, results from the exposure assessment were used the perform a ranking to direct risk reduction efforts (e.g. allocating resources) taking country-specific characteristics and behavior and prevalence of the hazard on the commodity into account.

7.4 Conclusion

Foodborne outbreaks associated with fruit and vegetables and border rejections generate economic losses and food waste and lead to loss of trust and confidence in the safety of fresh produce. From the current microbiological surveys conducted it could be established that fresh produce can overall be recognized as safe. However, during the PhD research there were some concerns noticed related to microbiological hazards, especially with regard to the presence of *Salmonella* on fresh herbs sourced or imported from other regions. Moreover, it was observed that both *Salmonella* and *E. coli* O157:H7 could survive on fresh herbs as basil during storage at different temperatures for one week. The survival of these pathogens during home storage, the fact that pathogens cannot be completely removed by washing and the predominantly raw consumption of fresh herbs indicate the importance of avoiding contamination during production and further in the food chain.

No *Salmonella* or STEC were detected on strawberries and thus strawberries were recognized as a safe fresh fruit commodity. However, a pressure from the production environment was observed with regard to potential introduction of pathogens, especially with regard to the irrigation water and the presence of farm animals close to the production area. This indicates that the awareness on transmission routes and GAP at primary production is needed, e.g. the use of fact sheets to communicate or stress biological hazards in self-checking guides. Moreover, training of workers (e.g. personal hygiene) and elaboration of appropriate check lists for competent authorities and traders/processing

companies to use in on-site assessment of production practices, may improve microbiological quality and safety of fresh produce being sold.

The GeneDisc Cycler PCR kit was able to detect genes of *Salmonella* and STEC that are present in low number on these fresh produce items. However, confirmation by isolation is deemed necessary. STEC was isolated from four environmental samples (irrigation water and substrate), but not from basil or strawberries. However, in eleven fresh herb samples and forty two environmental samples suspicious virulence genes, *stx* and *eae* genes, were detected by PCR. The complexity and still to some extent insufficient performance of the current isolation methods for STEC may be associated with an underestimation of STEC presence. On the other hand, detection of *stx* and *eae* may result in an overestimation. The additional PCR detection of a serogroup and its most likely associated *eae*-variant may reduce this overestimation. However, only a limited number of serogroups were included in the PCR analysis, whereby other serogroups able to cause disease will be missed. Further research should be conducted on an isolation procedure/differential media of STEC, especially for fresh produce commodities with a high level of background microbiota. Moreover, as it is still not possible to fully define human pathogenic STEC or identify factors for STEC that absolutely predict potential to cause human illness, further elucidation of virulence factors (or groups of virulence characteristics) of STEC is needed.

Results obtained in the present study indicate that an appropriate (multi) annual sampling plan for enteric pathogens as STEC and *Salmonella* has proven to be useful to gain insight in contamination routes and environmental pressure on pathogens' occurrence in the selected fresh produce items. Lessons learnt from the sampling and testing and the investigation of factors indicating higher likelihood of finding pathogens in the primary production may also help to establish better elaboration of guidelines for selection of (new) suppliers or trading partners for fresh produce and provide more trust in the quality and safety of fresh produce put on the market. However, as a control measure sampling and testing has its limitations and should be preferable combined and driven by knowledge obtained either during an inspection or audit on (systematic) noncompliance on good agricultural, hygienic or manufacturing practices, or information obtained during a sanitary survey on the production site indicating risk factors for human or animal fecal contamination. Still sampling and testing and setting of specifications is also a way of increasing awareness, highlighting the

point that presence of enteric pathogens such as *Salmonella* and STEC in fresh produce is not acceptable and thus helps to communicate the expected outcome of a production process and provides incentives to continually work on respect of good practices. Moreover, further investigation on a good surveillance system and reporting of foodborne outbreaks and outbreak investigation, should be developed.

By performing a quantitative risk ranking calculation, it was demonstrated that also niche products basil and strawberries can have an impact on public health equal or higher than more convenient consumed leafy greens. From the exposure model it was also derived that the prevalence was the main driving force, whereas the consumers' behavior influenced the outcome only to a minor extent.

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CURRICULUM VITAE

Stefanie Delbeke is born in Waregem on the 24th of March 1988. She obtained her high school degree in Science and Mathematics at Onze-Lieve-Vrouw Hemelvaart in Waregem in 2006 and graduated as a Master of Science in Bioscience Engineering: Food Science and Nutrition at Ghent University in 2011. Her master thesis, concerning the characterization of microbiological risks associated with the use of irrigation water in the plant production in Belgium, was completed at the Laboratory for Food Microbiology and Food Preservation. In August 2011, she started as a PhD researcher on the European project Veg-i-Trade at the same lab, under the guidance of Prof. dr. ir. Mieke Uyttendaele (promotor) and Prof. dr. ir. Liesbeth Jacxsens (co-promotor). The research was part of WP6: microbiological and risk analysis, with more in particular cases about *Salmonella* and STEC in strawberries and basil.

During the research she participated in various national and international conferences and published in international peer reviewed journals. Furthermore, she guided several students and assisted in the practical sessions of the Ghent University courses: Molecular Microbial Techniques, Food Microbiology and Food Preservation and Microbiological Safety and Analysis of Food. In the framework of this PhD research, she also cooperated with several farms and processing/trading companies for the sampling.

Stefanie Delbeke is geboren op 24 maart 1988 te Waregem. In 2006 behaalde zij het diploma Wetenschappen-Wiskunde aan het Onze-Lieve-Vrouw Hemelvaart in Waregem, vijf jaar later promoveerde zij tot Bio-ingenieur in de Levensmiddelenwetenschappen en Voeding aan de Universiteit Gent. De masterproef, omtrent de karakterisatie van microbiologische risico's verbonden aan het gebruik van irrigatiewater in de primaire plantaardige productie in België, werd volbracht aan het Laboratorium voor Levensmiddelenmicrobiologie en –conservering. In augustus 2011, startte zij als doctoraatsbursaal op het Europees project Veg-i-Trade op hetzelfde labo onder begeleiding van Prof. dr. ir. Mieke Uyttendaele (promotor) en Prof. dr. ir. Liesbeth Jacxsens (co-promotor). Haar onderzoek omkadert werkpakket 6: microbiologische analyse en risico analyse studies met als cases *Salmonella* en STEC in aardbeien en basilicum.

Tijdens dit onderzoek nam zij deel aan verscheidene nationale en internationale conferenties en publiceerde zij artikels in internationale tijdschriften. Tevens begeleidde zij verscheidene studenten en werkte mee aan de practica voor de vakken Moleculaire Microbiologische Technieken, Levensmiddelenmicrobiologie en –conservering en Microbiologische Veiligheid en Analyse van Levensmiddelen. Gedurende dit doctoraatsonderzoek werd nauw samengewerkt met aardbeitelers en bedrijven m.b.t. de verwerking en/of verhandeling voor de monstername.

Publications in A1 peer-reviewed journals

Delbeke, S., Ceuppens, S., Holvoet, K., Samuels, E., Sampers, I., Uyttendaele, M. (2015). Multiplex real-time PCR and culture methods for detection of Shiga toxin-producing *Escherichia coli* and *Salmonella* Thompson in strawberries, a lettuce mix and basil. *International Journal of Food Microbiology* 193:1-7. (DOI information: 10.1016/j.ijfoodmicro.2014.10.009)

Delbeke, S., Ceuppens, S., Jacxsens, L., Uyttendaele, M. Survival of *Salmonella* and *E. coli* O157:H7 on strawberries, basil and other leafy greens during storage. *Journal of Food Protection.* (DOI information: 10.4315/0362-028X.JFP-14-354)

Delbeke, S., Ceuppens, S., Hessel, C. T., Castro, I., Jacxsens, L., De Zutter, L., Uyttendaele, M. Microbial safety and sanitary quality of strawberry primary production in Belgium: risk factors for *Salmonella* and Shiga toxin producing *Escherichia coli* (STEC) contamination. *Applied and Environmental Microbiology* 18. (DOI information: 10.1128/AEM.03930-14)

(Submitted for publication in *International Journal of Food Microbiology*) **Delbeke, S.,** Ceuppens, S., Jacxsens, L., Uyttendaele, M. Microbiological analysis of pre-packed sweet basil (*Ocimum basilicum*) and coriander (*Coriandrum sativum*) leaves for the presence of *Salmonella* spp. and Shiga toxin-producing *E. coli*

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(In preparation) **Delbeke, S.,** Ceuppens, S., Johannessen, G., Allende, A., Sampers, I., Jacxsens, L. and Uyttendaele, M. Risk ranking of enteric pathogens *Salmonella* spp. and Shiga toxin-producing *E. coli* on fresh basil, strawberries and butterhead lettuce

Abstracts or posters on symposia

Delbeke, S., Jacxsens, L., Uyttendaele, M. (2014). Risk ranking of microbiological hazards *Salmonella* spp. and Shiga toxin-producing *E. coli* (STEC) on strawberry, butterhead lettuce and fresh herbs. Poster presentation. 19th Conference on Food Microbiology, 18-19 September, Brussels, Belgium.

Delbeke, S., Titze Hessel, C., Verguldt, E., De Beleyer, A., Clicque, T., Boussemaere, J., Jacxsens, L., Uyttendaele, M. (2014). Survival of *Salmonella* and *E. coli* O157 on strawberries and basil during storage at different temperatures. Poster presentation. 19th Conference on Food Microbiology, 18-19 September, Brussels, Belgium.

Delbeke, S., Titze Hessel, C., Verguldt, E., De Beleyer, A., Castro, I., Uyttendaele, M. (2013). Microbiological analysis of strawberries for the presence of *Salmonella* spp. and VTEC in primary production, processing and trade in Belgium. Poster presentation. 18th Conference on Food Microbiology, 12-13 September, Brussels, Belgium.

Delbeke, S., Holvoet, K., Samuels, E., Uyttendaele, M. (2012). GeneDisc multiplex-PCR and IMS-chromogenic media for detection of VTEC and *Salmonella* in lettuce, strawberries and basil. Poster presentation. FoodMicro, 3-8 September 2012, Istanbul, Turkey.

Delbeke, S., Holvoet, K., Samuels, E., Uyttendaele, M. (2012). GeneDisc multiplex-PCR and IMS-chromogenic media for detection of VTEC and *Salmonella* in lettuce, strawberries and basil. Poster presentation. 17th Conference on Food Microbiology, 20-21 September, Brussels, Belgium.

Delbeke, S., Holvoet, K., Samuels, E., Uyttendaele, M. (2012). GeneDisc multiplex-PCR and IMS-chromogenic media for detection of VTEC and *Salmonella* in lettuce, strawberries and basil. Poster presentation. IAFP European Symposium on Food Safety, 21-23 May, Warsaw, Poland.

Delbeke, S., Baert, L., De Keuckelaere, A., Li, D., Uyttendaele, M. (2012). Survival of norovirus, murine norovirus 1, MS2 phage and *E. coli* in various types of water used for irrigation of fresh produce. Poster presentation. 17th Conference on Food Microbiology, 20-21 September, Brussels, Belgium.

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De Keuckelaere, A., Baert, L., Duarte, A. M. M., **Delbeke, S**., Uyttendaele, M. (2011). Evaluation of viral concentration methods from processing water and different types of irrigation water using murine norovirus-1 and MS2. Poster presentation. 16th Conference on Food Microbiology, 22-23 September, Brussels, Belgium.

De Keuckelaere, A., Baert, L., Duarte, A. M. M., **Delbeke, S.**, Uyttendaele, M. (2011). Evaluation of viral concentration methods from processing water and different types of irrigation water using murine norovirus-1 and MS2. Poster presentation. VITAL Conference, 5-7 September, Ljubljana, Slovenia.

De Keuckelaere, A., Baert, L., Stals, A., De Vocht, M., Li, D., **Delbeke, S**., Lauryssen, S., Jacxsens, L., Sas, B., Uyttendaele, M. (2011). Survey conducted by a consumer organization for the presence of bacterial and viral pathogens on high risk fresh produce from the Belgian market. Oral presentation. 2nd International Conference on Quality Management of Fresh Cut Produce: Convenience for a tasteful life, 17-21 July, Torino, Italy.

De Keuckelaere, A., Baert, L., Stals, A., Li, D., **Delbeke, S**., Lauryssen, S., Jacxsens, L., Uyttendaele, M. (2011). Survey conducted by a consumer organization for the presence of bacterial and viral pathogens on high risk fresh produce from the Belgian market. Poster presentation. IAFP European symposium on food safety, 18-20 May, Ede, The Netherlands

Dissemination

Delbeke, S., Jacxsens, L., Uyttendaele, M. (2014). Allemaal beestjes, ook in de aardbeienteelt? Oral presentation. Veg-i-Trade feedback event to strawberry growers, December 11th, Roeselare, Belgium.

Delbeke, S., Jacxsens, L., Uyttendaele, M. (2014). Lusten *Salmonella* en Norovirus ook aardbeien? Oral presentation. Veg-i-Trade feedback event to strawberry growers, April 1st, Hoogstraten, Belgium.

Delbeke, S., Jacxsens, L., Uyttendaele, M. (2014). Microbiological analysis of strawberries and fresh herbs for the presence of *Salmonella* spp. and VTEC. Oral presentation. Veg-i-Trade meeting, January 2014, Porto Alegre, Brazil.

Delbeke, S., Uyttendaele, M. (2013). Microbiological analysis of strawberries and fresh herbs for the presence of *Salmonella* spp. and VTEC. Veg-i-Trade feedback event to Belgian and Dutch partners, June 11th, Gent, Belgium.

Delbeke, S., Jacxsens, L., Uyttendaele, M. (2012). Staalnameplannen in de keten van groenten en fruit: case microbiologie aardbeien en basilicum. Oral presentation. Veg-i-Trade: workshop veiligheid in de groenten en fruit keten: update en stand van zaken, March 8th, Lokeren, Belgium.

Delbeke, S., Jacxsens, L., Uyttendaele, M. (2012). Survival study: case strawberries and basil. Oral presentation. Veg-i-Trade: 4th consortium meeting, March 27th, Belgrade, Serbia.

Delbeke, S., Jacxsens, L., Uyttendaele, M. (2012). Screening strawberries and basil for VTEC and *Salmonella* spp.: GeneDisc Cycler. Oral presentation. Veg-i-Trade: 4th consortium meeting, March 27th, Belgrade, Serbia.

Doctoral schools

26-28 May 2014:	Module 10 'Nonparametric Methods' of the IPVW-course statistics (Specialized course)
5-6 December 2013:	Workshop/training in creative thinking (Transferable skills' training, cluster Career management)

13 – 24 August 2012:	BfR summer school on risk assessment and risk communication in Berlin (Germany) (Specialized course)
April/May 2012:	Effective scientific communication (cluster Communication skills)

Supervision of undergraduate students

Astrid De Beleyer (Bachelor paper: “De prevalentie en overleving van *Salmonella* spp. en VTEC op aardbeien”)

Tine Clicque (Internship paper: “Prevalentie en overleving van *Salmonella* spp. en VTEC op verse kruiden.”)

Jolien Boussemaere (Master dissertation: “Veiligheid en kwaliteit van verse basilicum”)