UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Assessment of Risk Factors Affecting the Microbial Safety of Leafy Greens in Spain

Evaluación del Riesgo Microbiano en la Producción y el Procesado de Hortalizas de Hoja

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2016

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2016

Table of contents

Summary

Fresh produce consumption has been increasing worldwide during the last years and together with this rise, food safety of fresh fruits and vegetables has become a major issue in the agro-food chain. Among fresh produce, leafy greens and particularly, salads ready to be consumed raw, have been recognized as a potential vehicle for transmission of pathogenic microorganisms known to cause human diseases. Major foodborne pathogens associated with these commodities are *Salmonella spp., Listeria monocytogenes* and pathogenic *Escherichia coli* strains. In the last decades, several reports have showed that enteric diseases linked to consumption of fresh produce have dramatically increased resulting in a concern for the public health as well as in economic losses to farmers, distributors and the food industry, in general. There are many risk factors that may contribute to the contamination of fresh-cut produce with human pathogens including weather factors, environmental factors, agricultural practices and hygiene practices among others. Thus, in order to reduce the microbial risks associated to leafy greens, the specific preventive measures and interventions should be evaluated and implemented.

The current thesis intended to provide an overview of the prevalence of the main microbial hazards commonly linked to leafy greens during production, harvesting and processing, as well as potential indicator microorganisms able to predict faecal contamination in leafy greens. The thesis was divided in seven chapters that are outlined in **Figure 1**. To introduce the research area, a literature review of the relevant aspects and the latest publications related to safety of leafy greens from the cultivation field to consumption was presented in *Chapter 1.*

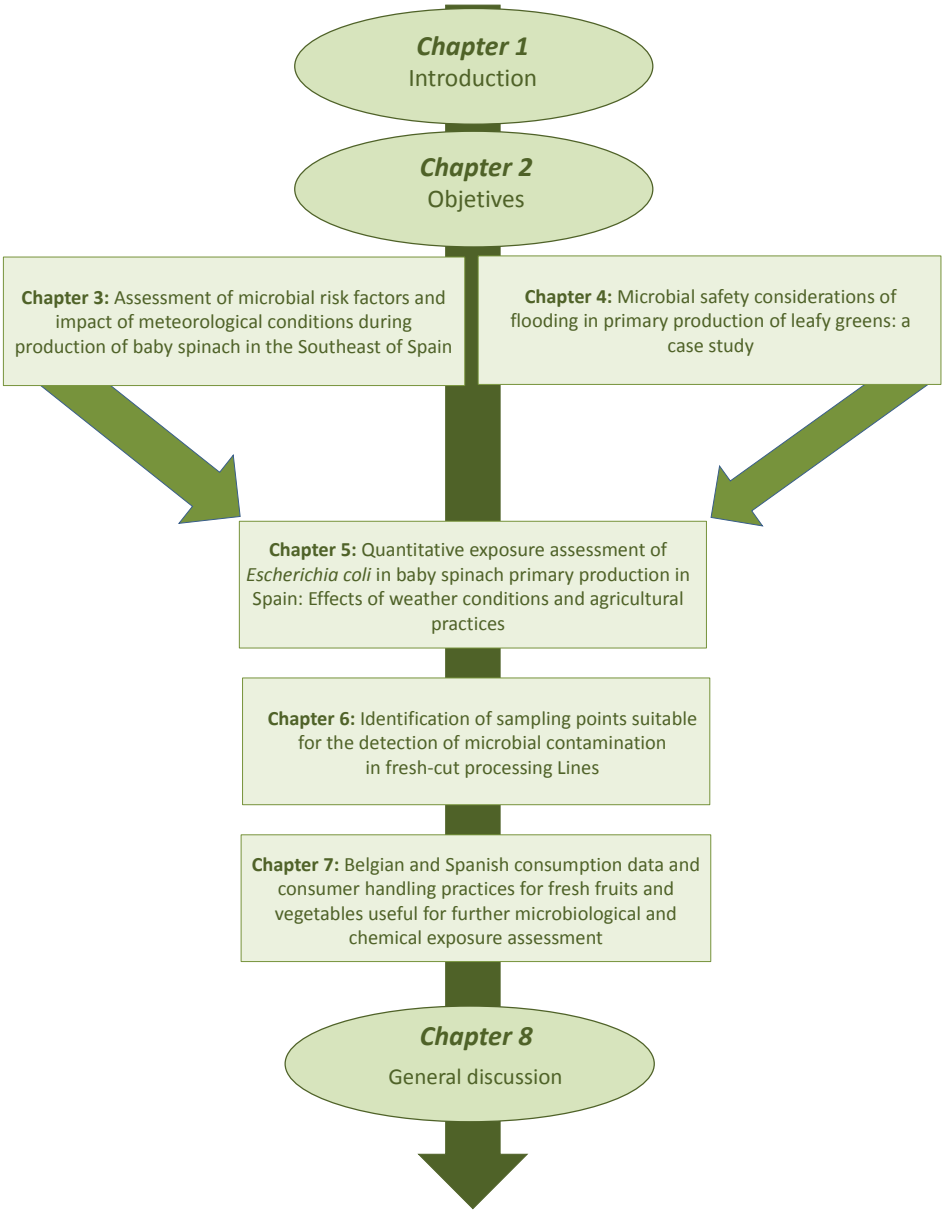

Figure 1. Outline of the dissertation.

Chapters 3, 4 and *5* were focused on the main factors affecting the microbial safety of leafy greens at primary production level. Firstly**,** *Chapter 3* evaluated the effect of agricultural factors and meteorological conditions on the microbial safety of leafy greens in Spain. In this study, soil and irrigation water were found to be the most important factors affecting the microbial safety of baby spinach. Concerning weather factors, it was suggested that ambient temperature affected the likelihood and extent the contamination of baby spinach. In relation to specific weather factors, *Chapter 4* studied the effects of extreme rainfall (i.e. flooding) on the

microbial safety of leafy greens together with the influence of subsequent weather parameters on the microbial persistence in soil, water and leafy greens. It was found that soil, irrigation and leafy greens samples taken from the floodplains after the event were contaminated with high levels of *E. coli* and pathogens (*Salmonella*, STEC and *L. monocytogenes*). However, a drastic decline was reported during the weeks after and it was attributed to climatic conditions after flooding, particularly the high solar radiation that affected the survival of bacteria in the fields. The main findings obtained in this *Chapter* confirmed previous research, which described flooding as a main risk factor for the microbial contamination of leafy greens and highlighted the need for mitigation strategies to protect production areas from incidents capable of releasing microbial contamination. It was also shown that climatic conditions, especially, solar radiation played an important role reducing the microbial survival on the crop.

In *Chapter 5* the mayor findings from chapters *3* and *4* together with a literature review of relevant publications were used to develop a quantitative microbial exposure assessment model (QMEM) of generic *E. coli* in baby spinach at primary production level in Spain. Once the model was built, it was used to evaluate the potential impact of weather factors (i.e. rain, solar radiation and simulation of a flooding event) and agricultural practices (i.e. irrigation method and water quality) on the distribution of generic *E. coli* levels on leafy greens at harvest. Jointly, our results confirmed that weather factors and management practices influenced the likelihood of leafy green contamination with *E. coli* and what intervention strategies aimed at such factors and practices could reduce the risk of pre-harvest contamination. Thus, QMEM was a useful approach to assess the potential impact of different risk factors and intervention strategies affecting *E. coli* concentrations at the field. Taking into account that generic *E. coli* strains may serve as a surrogate organism for enteric bacterial

pathogens, the results obtained on *E. coli* levels in baby spinach could be indicative of the potential behaviour of these pathogens under defined conditions.

Once the main risk factors for microbial contamination at field level were recognized, the next step was taken towards processing level. In this sense, *Chapter 6* was focused on microbial risks associated to the processing of leafy greens. The main purpose was to gain insight into the bacterial contamination throughout the operations involved in leafy green processing in order to identify critical sampling points and their relation with the microbial safety of end products. Samples of centrifuge water were found to be positive for both, pathogens and generic *E. coli*, which suggested that the origin of the contamination could be the produce subjected to centrifugation or the remaining process wash water. Further experiments confirmed the potential use of centrifuge water as a control point for detection of microbial contamination and opened future perspectives related to routine monitoring of this water as a critical place and sample for the evaluation of product safety.

In *Chapter 7*, the focus was made on the last step of the farm-to-fork chain: consumers' level. Firstly, the suitability of exiting data on the consumption of fresh fruit and vegetables was evaluated and it was established that detailed information about actual consumption of different types of fresh produce and the corresponding handling practices used by consumers at home was lacking. Therefore, this chapter was intended to provide standardized data for fresh produce consumed raw or minimally processed that could be used in future exposure assessments related to fresh produce (i.e. RTE leafy greens).

In *Chapter 8*, the main results of this thesis together with the future needs and perspectives resulting from this work were discussed.

Resumen

En los últimos años, el consumo de productos frescos ha aumentado de manera global y, en consecuencia, la seguridad alimentaria de frutas y hortalizas frescas se ha convertido en un asunto importante en la cadena agroalimentaria. Entre los productos frescos, las hortalizas de hoja y en particular, las ensaladas listas para consumir, han sido identificadas como un vehículo potencial para la transmisión de microorganismos patógenos humanos. Los principales agentes patogénicos transmitidos por los alimentos asociados con estos productos son las cepas patógenas de *Salmonella* spp., *Listeria monocytogenes* y *Escherichia coli*. En las últimas décadas, se ha producido un importante incremento de las enfermedades entéricas ligadas al consumo de productos frescos lo que resulta no sólo en un problema para la salud pública, sino también en un problema de pérdidas económicas para agricultores, distribuidores y para la industria alimentaria, en general. Son muchos los factores de riesgo que pueden contribuir a la contaminación de los productos listos para consumo con patógenos humanos, estos incluyen factores climáticos, factores ambientales, prácticas agrícolas y de higiene entre otros factores. Por lo tanto, con el fin de reducir los riesgos microbianos asociados al consumo de hortalizas de hoja, es importante contar con estrategias de mitigación y prevención de la contaminación microbiana, tales medidas deben ser a su vez evaluadas para después ser posteriormente puestas en práctica.

La presente tesis está destinada a proporcionar una visión general de los principales riesgos microbiológicos asociados a las hortalizas de hoja durante su producción, cosecha y procesado, así como los posibles microorganismos indicadores capaces de predecir o dar una estimación de la posible contaminación con microorganismos patógenos en hortalizas de hoja. Para introducir el área de estudio, el primer capítulo proporciona una revisión bibliográfica de

aquellos aspectos más relevantes así como de las últimas publicaciones relacionadas con la seguridad microbiológica de las hortalizas de hoja desde el campo de cultivo hasta su consumo.

Los *capítulos 3, 4* y *5* se han centrado en los principales factores que afectan a la seguridad microbiana de las hortalizas de hoja a nivel de producción primaria. En primer lugar, en el *Capítulo 3* se evaluó el efecto de los factores agrícolas y las condiciones meteorológicas en la seguridad microbiana de hortalizas de hoja en España. En este trabajo, se identificaron el suelo y el agua de riego como los factores más importantes que afectan a la seguridad microbiana de la 'espinaca baby' en el campo de cultivo. En cuanto a los factores climáticos, se observó que la temperatura ambiente afectaba la probabilidad de la contaminación de la 'espinaca baby'. Con respecto a factores climáticos más específicos, el *Capítulo 4* se estudiaron los efectos de las precipitaciones extremas (es decir, inundaciones) sobre la seguridad microbiana de hortalizas de hoja. También se estudió la influencia del tiempo de espera tras la inundación sobre la persistencia de los microorganismos en el suelo, el agua y las hortalizas de hoja. Se encontró que las muestras de suelo, el agua de riego y hortalizas de hoja tomadas de campos afectados por la inundación estaban contaminados con altos niveles de *E. coli* y patógenos (*Salmonella* spp., STEC y *L. monocytogenes*). Sin embargo, se observó un drástico descenso de dicha contaminación en las semanas posteriores. Este descenso se atribuyó a las condiciones climáticas registradas tras las inundaciones, en particular la alta radiación solar que afecto a la supervivencia de los microorganismos en el campo de cultivo. Los resultados obtenidos en este capítulo corroboraron estudios previos que identificaban las inundaciones como importantes factores de riesgo para la contaminación microbiana de hortalizas de hoja confirmando la necesidad de estrategias de mitigación para proteger las zonas de cultivo de este tipo de incidentes capaces de liberar y dispersar contaminación microbiana. También se puso de manifiesto que las condiciones climáticas, sobre todo, la radiación solar jugaron un papel importante reducción de la supervivencia microbiana en el cultivo.

En el *Capítulo 5* los principales hallazgos de los *Capítulos 3 y 4*, junto con una revisión bibliográfica de las publicaciones más relevantes se utilizaron para desarrollar un modelo de análisis cuantitativo de riesgo microbiológico para la evaluación de la exposición microbiana de *E. coli* en 'espinaca baby' al nivel de producción primaria en España. Una vez que el modelo fue construido, se usó para evaluar el impacto potencial de diferentes factores microbiológicos (precipitaciones, radiación solar y la simulación de una inundación) y prácticas agrícolas (método de riego y la calidad del agua) en la distribución de los niveles de *E. coli* en hortalizas de hoja en el momento de la cosecha. En conjunto, los resultados confirmaron el impacto tanto de los factores climáticos como de las prácticas agrícolas en la contaminación de las hortalizas de hoja con *E. coli*. En este capítulo también se puso de manifiesto que la aplicación de medidas preventivas dirigidas específicamente a dichos factores y prácticas de riesgo podría reducir el riesgo de contaminación antes de la cosecha. Por lo tanto, el modelo resultó un método útil para evaluar el impacto potencial de los diferentes factores de riesgo y las estrategias de intervención que afectan a las concentraciones de *E. coli* en el campo de cultivo. Teniendo en cuenta que las cepas genéricas de *E. coli* pueden servir como un microorganismo tipo para bacterias patógenas entéricas, los resultados obtenidos en los niveles de *E. coli* en 'espinaca baby' podría ser indicativo del comportamiento potencial de estos patógenos en los campos de cultivo.

Tras la identificación de los principales factores de riesgo de contaminación microbiológica a nivel de campo, el paso siguiente se dirigió hacia el siguiente paso en la cadena, es decir, la planta de procesado de IV gama. Siguiendo esta línea, el *Capítulo 6* se centró en los riesgos microbiológicos asociados al procesado de hortalizas de hoja. El principal objetivo fue obtener una visión global de la contaminación bacteriana en todas las operaciones que implica el procesado de hortalizas de hoja con el fin de identificar los puntos críticos de muestreo y su relación con la seguridad microbiológica del producto final. En este sentido, se

encontró que las muestras de agua obtenidas de la centrifuga eran positivas tanto para *E. coli* genérica como para microorganismos patógenos, sugiriendo el posible origen de la contaminación en el producto sometido a centrifugación o en el agua de lavado. Además, se llevaron a cabo otros experimentos que confirmaron el uso potencial de agua centrífuga como punto de control para la detección de contaminación microbiana abriendo perspectivas futuras relacionadas con el seguimiento de la calidad microbiológica de este agua para la evaluación de seguridad microbiológica del producto final.

En el *Capítulo 7*, la atención se centró en el último paso de la cadena del 'campo a la mesa': el consumidor. En primer lugar, se evaluó la validez de los datos existentes referentes al consumo de frutas y hortalizas frescas en España y Bélgica, llegando a la conclusión de que la información disponible sobre el consumo real y detallado de diferentes tipos de productos frescos así como las prácticas de manipulación de los mismos era deficiente. Por lo tanto, en este capítulo el objetivo fue proporcionar datos estandarizados para el consumo de productos frescos destinados a ser consumidos crudos o mínimamente procesados. Estos datos podrían ser de gran utilidad en futuros estudios de evaluación de la exposición a diferentes riesgos tanto químicos como microbiológicos con productos frescos listos para el consumo (por ejemplo, hortalizas de hoja).

En el *Capítulo 8*, se discuten los principales resultados de esta tesis, junto con las futuras necesidades y perspectivas para investigaciones futuras que resultan de este trabajo. Por último, en el *Capítulo 9* se establecen unas breves conclusiones sobre los principales resultados obtenidos en el transcurso de estas investigaciones.

List of abbreviations

AA	Aggregative adherence
ANOVA	Analysis of Variance
Aprox.	Approximately
ºC	Degree Celsius
CAC	Codex Alimentarius Commission
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
Ca^{2+}	Calcium ion
$Ca(ClO)_2$	Calcium hypochlorite
CECT	Spanish Type Culture Collection
CFU	Colony Forming Units
CDC	Centres for Disease Control and Prevention
CDHS	California Department of Health Services
CEBAS	Centro de Edafología y Biología Aplicada del Segura
CI	Confidence interval
cl	centilitre
ClO_2	Chlorine dioxide
cm	Centimetre
cm^2	square centimetre
CO_2	Carbon Dioxide
CSIC	Spanish National Research Council
CSL	Critical sampling location
CT-SMAC	Cefixime Tellurite Sorbitol MacConkey
d	Day(s)

DAEC	Diffusely Adherent *E. coli*
DBPs	Derived by-products
DNA	Deoxyribonucleic acid
Eae	Attaching and effacing gene
EAEC	Enteroaggregative *E. coli*
EAHEC	Enteroaggregative Hemorrhagic *E. coli*
EC	European Commission
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
e.g.	'for example'
EHEC	Enterohemorragic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPA	Environmental Protection Agency
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxinogenic *E. coli*
EU	European Union
EUFIC	European Food Information Council
FAO	Food and Agricultural Organization
FAOSTAT	Statistics Division of Food and Agricultural Organization
FDA	Food and Drug Administration
FEPEX	Spanish Federation of Associations of Producers and Exporters of Fruits, Vegetables, Flowers and Live Plants
FFQ	Food frequency questionnaires
FoNAO	Food of non-animal origin
FSANZ	Food Standards Australia & New Zealand

FSMS	Food Safety Management System
g	Gram(s)
G	Genogroup
GAP	Good Agricultural Practices
GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices
h	Hour
H_2O	Water
HACCP	Hazard Analysis and Critical Control Points
Hg/ha	Hectogram/hectare
i.e	'that is'
IFS	International Food Standard
ISO	International Standards Organization
HuNoVs	Human noroviruses
HUS	Haemolytic-uraemic syndrome
IFPA	International Fresh-Cut Produce Association
IMS	Immunomagnetic separation
IPCC	Intergovernmental Panel on Climate Change
iroB	Fur-regulated gene of *Salmonella*
kG	Kilogram
L	Litre
lab	Laboratory
log	Logarithm
m	Metre(s)
m^2	Square metre(s)

MAP	Modified Atmosphere Packaging
max	Maximum
mg	Milligram(s)
µg	Microgram(s)
µm	Micrometre(s)
min	Minute(s)
MJ	Megajoule
mL	Millilitre(s)
mm	Millimetre(s)
n	Number of samples
N	North
NoV	Human norovirus
NaCl	Sodium chloride
NaClO	Sodium hypochlorite
NA	Not analysed
O_2	Oxygen
PCA	Plate Count Agar
PCR	Polymerase chain reaction
pH	Power of hydrogen
PHE	Public Health England
%	Percentage
QMEM	Quantitative Microbiological Exposure Model
QMRA	Quantitative Microbiological Risk Assessment
RH	Relative humidity
RTE	Ready-to-eat

RT-PCR	Real Time - PCR
s	Seconds
spp.	Species
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxin-coding gene
T	Time
THMs	Trihalomethanes
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
VTEC	Verotoxin producing *Escherichia coli*
W	West
WHO	World Health Organization
XLD	Xylose-Lysine-Desoxycholat

Tables & Figures

Chapter I: Introduction

Chapter I

Leafy greens are, together with fruit and other vegetables important components of a healthy and balanced diet and its consumption is being encouraged in many countries, including Spain, by government health agencies (MAGRAMA, 2015). However, fresh produce, and in particular leafy greens that are usually consumed raw, are increasingly being recognized as important vehicles for transmission of human pathogens. There is currently limited knowledge about where in the supply chain contamination occurs and the mechanisms by which human pathogens colonize and survive on leafy greens once contaminated. This thesis is focused on the microbial hazards linked to leafy greens production, harvesting and processing. In order to introduce the research area, a literature review on the relevant aspects and latest publications related to leafy greens from production to consumption was carried out in the present chapter.

1. Leafy greens

Leafy greens, also called leafy vegetables, include all vegetables of a leafy nature and of which the leaf (and core) is intended to be consumed raw, e.g. lettuce (all varieties), spinach, cabbages, chicory, and watercress among others (FAO/WHO, 2008). A more detailed definition provided by the European Food Safety Agency (EFSA) includes: beet greens, bitterleaf, bok choy, cabbage, celery, celtuce, Ceylon spinach, chard, chicory, Chinese cabbage, collard greens, cress, endive, epazote, garden cress, garden rocket, komatsuna, lamb's lettuce, land cress, lettuce, mizuna greens, mustard, New Zealand spinach, pak choy, radicchio, rapini, spinach, tatsoi, watercress, water spinach and wrapped heart mustard cabbage among others In general, this group encompasses a wide and continuously changing assortment of species and varieties (EFSA, 2013).

The market for freshly prepared vegetable products has increased explosively during the last years (Leon et al., 2009; Betts, 2014). The main driving force for this

market growth is the increasing consumer demand for fresh, healthy, convenient and additive-free prepared products (FAO, 2010). In Spain, this market is still in an early stage of development but recently it has shown a continuous growth, showing an annual sale increase of 5-6 %, with 70.600 and 74.064 tons in 2010 and 2011, respectively (Anonymous, 2013). After this period, the Spanish market stabilized to approximately 77.000 tons sales in 2013 (Anonymous, 2014).

Consumers' expectation of year around availability of fresh products has encouraged the globalization of food markets (Yu & Nagurney, 2013). Consequently, fresh produce is grown and exported in large volumes; as a result, its production chain is becoming more complex and globalized (Jacxsens et al., 2010; Yu & Nagurney, 2013). Additionally, large volumes of fresh produce are traded on the international market originated from diverse climatic and geographical regions (Ongeng et al., 2015). Thus, the globalization of the fresh produce supply chain has resulted in unique food safety challenges that may affect fresh produce safety (Jacxsens et al., 2010). For instance, globalization can led companies to purchase raw materials from suppliers located in developing countries which increases the chance of unknown hazards and unexpected contamination. Many of these countries have basic safety standards or do not comply with internationally accepted requirements (Kirezieva et al., 2013a,b).

The European Union produces about 50 million tons of vegetables yearly and it is the world's main importer of fruits and vegetables (FAOSTAT, 2015). The quantities produced for the above mentioned leafy vegetables vary widely among EU countries. Though there are not suitable data for all the member states of the EU, the available annual data for Member States showed that between 2007 and 2012, the EU production of lettuce was between approximately 2 million and 2.5 million metric tons per year (EFSA, 2014a).

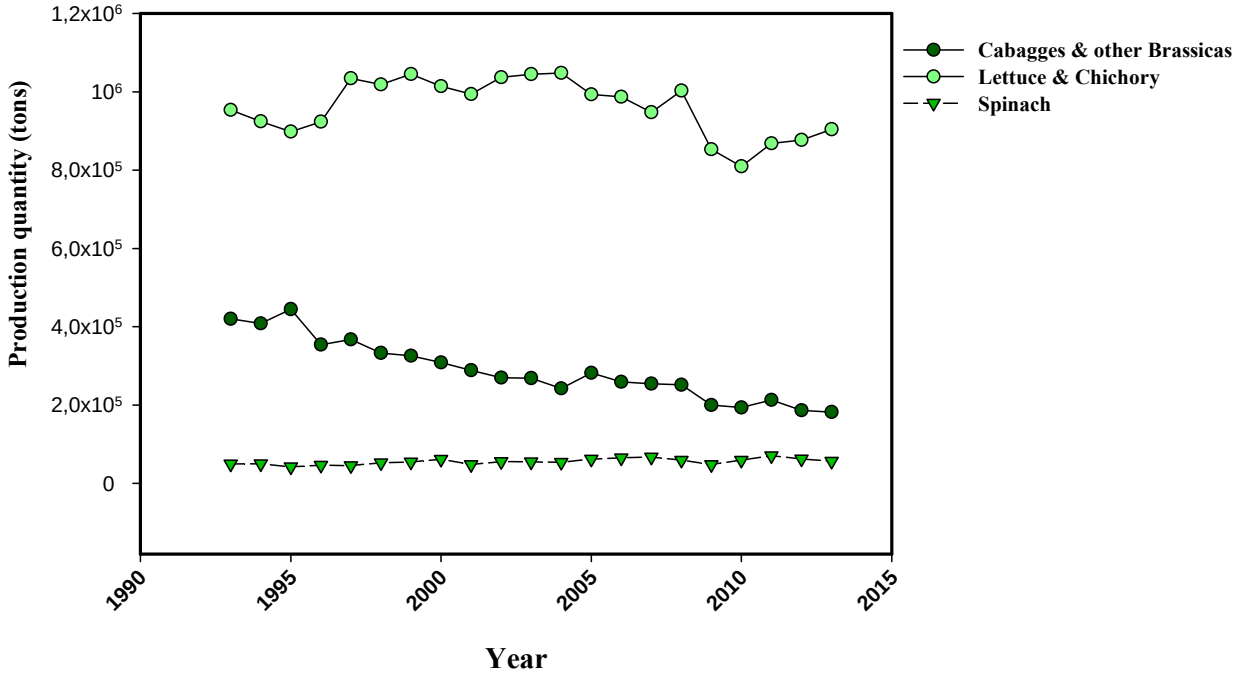

Figure 1.1. Annual amount (tons) of three leafy greens commodities produced in Spain (FAOSTAT, 2015).

In Spain, information on production variables for available leafy greens commodities were performed using existing data between 1993 and 2013 from the FAOSTAT database (http://faostat.fao.org). The production of some leafy greens in Spain has been stable during the last two decades (**Figure 1.1**). Lettuce and chicory represent the highest production with 904300 tons in 2013. Production of spinach is significantly lower (56.700 tons in 2013) but it constantly increased the last years (FAOSTAT, 2015). In Spain, the RTE salad industry is currently represented by three major industries: Florette Ibérica, Verdifresh and Primaflor (Alimarket, 2014). Florette Ibérica was the first Spanish company to produce bagged salads. This company started with a processing plant in Milagro (Navarra), the first of five plants built around the country since 1989. Florette Ibérica has been catalogued as the "leader company" in Spain because it has around 1,800 hectares of cultivation fields and produces 39.000 tons of salads and greens per year (El País, 2015).

Table 1.1. Total production and economic value of exported leafy greens (FEPEX, 2015).

	Total production (tons)			Economic value (Euros)		
Country	***Endive & Chicory***	***Spinach***	***Lettuce***	***Endive & Chicory***	***Spinach***	***Lettuce***
Germany	5.200	983	123.869	4.716.224	1.580.359	107.275.165
Austria	566	24	7.956	615.220	27.200	8.142.105
Belgium	751	321	5.457	919.087	600.163	5.232.541
Bulgary	40	3	1.531	51.425	4.449	1.395.347
Cyprus	90	-	404	77.280	-	359.505
Croatia	-	1	342	-	672	317.160
Denmark	256	214	10.492	324.693	259.864	10.070.502
Slovaquia	263	-	1.244	261.824	-	998.881
Slovenia	39	-	229	33.186	-	210.254
Estonia	194	0	79	139.796	608	83.374
Finland	96	40	8.512	73.114	121.326	7.226.618
France	16.941	2.982	69.015	11.753.397	2.654.070	61.702.145
Greece	39	19	522	42.759	3.655	672.777
Hungary	163		3.596	126.272	-	3.256.931
Ireland	160	248	2.835	153.445	317.466	2.318.063
Italy	5.188	6	34.541	4.117.430	6.564	25.450.580
Latvia	39	6	713	59.500	9.761	736.505
Lithuania	303	6	4.182	284.598	12.501	3.692.196
Luxemburg	21	-	30	36.918	-	56.536
Malta	-	-	263	-	-	270.932
The Netherlands	7.769	3.603	41.559	7.049.100	5.393.629	33.765.168
Poland	3.595	423	18.309	3.114.462	587.776	15.282.884
Portugal	386	101	1.142	493.924	148.732	1.046.204
United Kingdom	2.754	7.283	79.083	3.108.398	13.058.456	70.316.635
Czech Republic	381	7	7.652	422.119	13.274	6.312.366
Romania	244	-	426	213.588	-	405.169
Sweden	334	528	19.667	308.336	965.935	19.404.780
TOTAL EU-28*	**45.812**	**16.798**	**443.650**	**38.496.095**	**25.766.460**	**386.001.323**
EXTRA EU**	**1.934**	**182**	**21.104**	**1.653.569**	**407.262**	**23.937.166**
TOTAL	**47.746**	**16.980**	**464.754**	**40.149.664**	**26.173.722**	**409.938.489**

*TOTAL EU-28: refers to transactions within the EU 28 member countries. ** EXTRA-EU refers to transactions with all countries outside of the EU.

Exportation is also important in the Spanish leafy green sector. Within Spain, the main productive regions are Andalucía, Valencia and Murcia. **Table 1.1** represents the total amount (tons) and total value (euros) of the major leafy greens commodities (i.e. endive and chichory, spinach and lettuce) exported from Spain to the rest of EU countries during the first semester of 2015 (FEPEX, 2015).

2. Production chain of leafy greens

In EU, there is a wide diversity of practices at all stages of production including, type of irrigation, substrates (soil, hydroponic) and settings (open air, greenhouses, tunnels or production rooms) among others (EFSA, 2014a). Once processed, a wide range of cutting, washing, packaging and storage conditions are also used within this industry. Commonly, leafy greens for the fresh-processing industry follow a well-defined process (FDA, 2008; EFSA, 2014a). However, the range of leafy greens varieties is extensive and therefore the practices associated with their production and processing vary widely among them.

2.1 European and Spanish primary production of leafy greens

Production takes place all over the EU depending on the season although the biggest producers are located in Spain, Italy, France and Germany. The main species produced are *Lactuca sativa*, *Cichorium endivia*, *Beta vulgaris*, *Valerianella locusta*, *Cichorium intybus*, *Eruca vesicaria* subsp. *sativa, Spinacea oleracea, Brassica rapa, Brassica oleracea* and *Nasturtium officinale*. Apart fromr *S. oleracea* (spinach), *C. intybus* (Belgian endive) and *Brassica* spp. (cabbage), these leafy greens are mostly consumed fresh and fresh-cut prepared (Callejón et al., 2015).

In Spain, most of RTE products fall into two categories: mature-leaf salads - such as lettuces, curly endives and greens – and baby-leaf salads, introduced into Spain by Florette. Mature-leaf salads are grown outdoors, while baby-leaf salads are often cultivated in plastic-sheeted greenhouses because they are more delicate. In the case of Florette Ibérica, mature-leaf salads are cultivated in 1.400 hectares while baby leaves are in almost 400 hectares (El País, 2015).

'Lettuce'-type leafy greens can be harvested at different physiological states, e.g. mature whole heads, baby leaves and as multi-leaves. Mature whole heads, such as iceberg lettuce, are harvested when heads have developed the appropriate density and market size while baby leaves (e.g. baby spinach) are collected at an immature stage. In case of whole heads, the production cycle lasts from 4 to 15 weeks, while baby leaf cycles are usually shorter, lasting about 4-6 weeks from sowing to harvest depending on the season (Lestrange, 1999; EFSA, 2014a). Baby leaves are planted and grown similarly to standard varieties of whole heads with the exception of the plant density. Baby leaves are physically smaller than whole heads. For baby leaves, sowing is usually performed directly on beds using a very high plant density of 800 plants m^2 (Selma et al., 2012). Production of seeds for leafy greens production involves different pre-harvest activities such as field preparation, sowing, growth (including flowering and seed setting), irrigation, fertilization, pollination, swathing, field drying, seed harvest, storage and transport (FSANZ, 2010). Plants for seed production are grown in typical agricultural environments and seeds are generally treated as raw agricultural products. A wide range of seeds is used for leafy greens and thus a diverse range of agricultural practices may be associated with their production (FSANZ, 2010). Once seeds are harvested, they have to be processed in order to remove foreign material such as soil, weed seeds, insects and other debris. The cleaning step is performed by passing the seeds through a series of sieves and then further sorting via use of a gravity table, where seeds are separated by weight. The process may reduce, but it is unlikely to eliminate pathogenic microorganisms if present on or in the seed coat (EFSA, 2014a). Once cleaned, seeds are generally packed into bags for the bulk seed market. Seed companies recommend maintaining seed stocks in conventional refrigerators if possible, or stored at lower temperatures than 15 ºC (RijkZwaan, 2005).

As mentioned before, leafy greens can be cultivated in both open fields and greenhouses. In Spain, the predominant production method is open field although some are produced in greenhouses (El País, 2015). The crops are grown in a bed system and most of soil types are suitable for leafy greens although the highest production is obtained on fertile loams that are rich in organic matter (Lestrange, 1999; Enza Zaden, 2013). The first step of the primary production is the preparation of the soil for planting which consist of fertilization aimed to optimize the crop quality. Chemical and/or organic fertilizers can be used (EFSA, 2014a). Chemical fertilizers are usually used because they are easy to transport, improve efficiently for plant growth and give high yields. However, it has been observed that with successive cultivation, the quantity of chemical fertilizers has to be increased because of the declining in soil fertility (Edmeades, 2003). Regarding organic fertilizers, manure is an excellent source of crop nutrients that improves soil structure through provision of organic matter (Masse et al., 2011). For the production of vegetables, animal manure is commonly used as fertilizer in agriculture (Franz et al., 2008a; Johannessen et al., 2005). Manure products (including those from all farmed animals such as cattle, poultry, etc.) used for fertilization should be treated to inactivate pathogenic microorganisms (EU, 2011). According to UK guidelines, if fresh solid manure or slurry is applied to the field, harvest cannot take place within 12 months. A waiting time of at least 6 months is advised before drilling or planting. If treated or batch stored solid manure or slurry is added to the land, there is no waiting time necessary (Food Safety Agency UK, 2009). After applying manure, the land is tilled with a cultivator to prepare the field for sowing. For sowing, leafy green seeds are usually direct drilled into beds at a specified density, but recently there has been an increase in transplantation of seedlings which are produced in greenhouses or tunnels (Enza Zaden, 2013).

In relation to irrigation water and method, both depend on the soil type and climatic conditions. Water from diverse sources (e.g. collected rainfall, subsurface, surface, or reclaimed water) has been used in the production of leafy greens (Uyttendaele et al., 2015). Many irrigation methods (e.g. drip irrigation, overhead sprinkler, furrow, sub-irrigation systems) can be chosen to maintain a good availability of water for the crop (Nicola et al., 2009). In Europe, the major irrigation systems used in agricultural production are drip and sprinkler irrigation (EFSA, 2014a; Uyttendaele et al., 2015). Sprinkler irrigation offers several advantages over surface irrigation methods, such as higher water use efficiency, better fertilizer application and high yield although it cannot be applied when higher wind velocities occur (Camp et al., 2001; Tagar et al., 2012). Furrow irrigation is a surface irrigation system that can be found in small-scale farms because it does not require high investment in equipment. Drip irrigation applies water directly to the root zone of plants and its major advantages over sprinkler and furrow irrigation include: saving of water, increased efficiency of fertilizer use, reduced energy consumption and tolerance of windy conditions (EFSA, 2014a; Monaghan & Hutchison, 2012). During the production process of leafy greens, water plays an essential role from the start of the production process as a carrier of pesticides and nutrients for the growth of the crops. During the growing period, irrigation is frequently applied depending upon the temperature and amount of precipitation (EFSA, 2014a). Physical and chemical disinfection systems have been explored as methods to remove human pathogens from agricultural water sources although disinfection treatment of irrigation water is still a very limited practice (Suslow, 2015). Nowadays, chemical sanitizers are the most commonly used water treatments, although environmentally friendly alternatives are being demanded, particularly for organic production (Allende & Monaghan, 2015). Among commercially available water treatments, chlorine-based sanitizer is the most common choice for the removal of

biohazards from irrigation water (Gil et al., 2015; Suslow, 2015). Two of the more widespread treatments are calcium hypochlorite and chlorine dioxide (Allende & Monaghan, 2015). However, they have some limitations in terms of formation of disinfection by-products, which may have a potential negative effect on the environment and limited its use in agricultural water (FAO/WHO, 2009b). Consequently, alternative greener technologies based on physical treatments such as ultrasound (US), ultraviolet light (UV-C) and filtration systems have been successfully tested (Jones et al., 2014; Suslow, 2015; Villanueva et al., 2015) and put into practice by growers. Generally, these technologies have the advantages of not generating disinfection by-products and being relatively cheap (Villanueva et al., 2015). Additionally, innovative filtration systems containing sand and/or materials with reactive components have been explored as potential water treatment such as bio-sand filter zero-valent iron incorporated (ZVI) treatments that have been recently proposed as a cost-effective mitigation strategy for irrigation water treatment to reduce *E. coli* infections with contaminated leafy greens (Ingram et al., 2012).

When leafy greens are mature, they are manually or mechanically harvested. Mechanical harvest is faster than hand harvesting, but depending on the crop, it can result in significant damage to the produce (Fallon et al., 2011). Mechanical damage during harvest can become a serious problem, as plant injuries predispose produce to decay, increased water loss as well as increased respiratory and ethylene production rates which can accelerate deterioration and internalization and proliferation of microbiological contamination (Kitinoja & Kader, 2002). Manual harvesting is still an often practice for whole heads. The product is separated from the plant roots and manually removed from the growth substrate by using a knife or clipper (Suslow et al., 2003; EFSA, 2014a). Some fresh-cut processors use field coring and trimming of lettuce (Taormina et al., 2009). After harvested, leafy greens (mostly whole lettuce heads) may be directly packaged by hand in

the field, which is usually assisted by a variety of equipment that includes conveyors and mobile packing stations and transported to the industry for further processing (EFSA, 2014a).

The handling conditions between harvest and processing of leafy greens are critical to maintain the quality and safety of the product (Francis et al., 2012). In fact, guidelines recommend that leafy greens must be cooled as soon as possible in order to preserve quality (Thompson et al., 2002) and restrict growth of foodborne bacterial pathogens (Matthews, 2014). However, this practice is not always applied by growers (Rogers et al., 2006; Thompson et al., 2008). The recomended temperature to maintain leafy greens quality as long as possible is between 0 and 5 ºC (EFSA, 2014a). Different cooling systems have been recommended such as the use of cold rooms, forced-air cooling, vacuum cooling and hydro vacuum-cooling (Thompson et al., 2008). In the case of Florette Ibérica and most of RTE salad producing industries, the processing plants are located near the fields where the leafy greens are grown. Consequently the raw material arrives quickly to the processing industry. Once there, this raw material is normally stored in a cold-storage room that maintains a temperature of no more than 4 ºC (El País, 2015).

2.2 European and Spanish processing of leafy greens

Leafy greens may be further processed to obtain ready-to-eat products. Processing steps include: selection, elimination of external leaves, cutting, washing, rinsing, dewatering, packaging and storage (EFSA, 2014a). Commonly, the extension of the shelf life depends on a combination of proper temperature management throughout the entire cold chain, dips in anti-browning solutions, optimal packaging conditions and good manufacturing and handling practices in well-designed factories (Artés et al., 2009). The main objective of the fresh fruit and vegetable processors throughout all processing

operations involved in the production of fresh-cut produce is food safety and quality optimization (Osterholm et al., 2009).

In general, the first step is the reception and inspection of the raw material to assure the quality of the product, particularly when harvested leafy green have undergone mechanical damage which may cause internalization and proliferation of microbiological contamination (Aruscavage et al., 2008; Brandl, 2008). Following this selection, high quality product is stored under refrigeration conditions, and processing will vary depending on the type of product (EFSA, 2014a). The temperature in the processing plant is usually maintained between 5 to 10 ºC during storage prior to processing (Spanish Companies information).

For whole heads, external leaves and the core, if not removed in the field, are manually removed. Hand knives and stationary coring units are used for this operation (Suslow et al., 2003). The other parts of the lettuce are cut or shredded to pieces of different sizes, which might vary between 1 to 6 cm in size, using industrial rotary stainless steel blades. When baby leaves or multi-leaves are processed, steps such as the elimination of external leaves and cores are not needed, and in most instances, these types of products start their processing in the pre-washing or washing step. Washing is carried out to remove dirt, foreign materials, tissue fluids from cut surfaces, and microorganism and this is the only processing step that reduces the microbial load on the lettuce (Gil et al., 2009).

Washing can be achieved by simply spraying with potable water, although it generally involves the immersion of the product in washing tanks with chilled water (1 to 5 ºC). In case of Spanish RTE industries, disinfectants are always added to the washing tanks (Spanish Companies Information). In the case of other European countries, sanitizer use depends upon national policies (Gil et al., 2009). Sanitizers are used to maintain the

microbial quality of the water, which is needed to avoid cross-contamination between different lots (EFSA, 2014a). Among available water disinfectants, chlorine is generally the most commonly sanitizer used in leafy green processing due to the low cost, the reliable availability, the good effectiveness against suspended vegetative bacteria and some enteric viruses, and the minimal impact on the nutritional and sensorial quality of the produce (Tomás-Callejas et al., 2012; Gil et al., 2015). The election of the sanitizer, concentration dose and the mode of washing vary depending on the processor (EFSA, 2014a). As an example chlorine at 20-40 mg free chlorine per litre may be used when washing tanks are used. In this case, the temperature of the water is usually maintained between 4-5 ºC, contact times are from 1 to 3 min and pH values vary between 6 and 7 to ensure the presence of chlorine in the hypochlorous acid form and minimize corrosion of equipment (FAO, 2003; Van Haute et al., 2013; Gil et al., 2015). Nevertheless, although chlorine is the most commonly used sanitizer its potential impact on human health and the release of hazardous by-products into the environment have hastened the research for alternative, safe and cost-effective sanitizers. Optimization of equipment design and processing practices for washing and sanitizing are essential for increasing microbial reduction and ensuring microbial safety of fresh vegetables (Matthews, 2013).

If a sanitizer is not used, processing of leafy greens relies on continuous addition and refreshing of washing baths with large volumes of potable water, up to 40 l/kg of raw produce, to minimize the accumulation of organic matter and subsequently microorganisms, in the water and transfer of microorganisms from the water to the fresh-cut leaves (Selma et al., 2008; Olmez & Kretzschmar, 2009). In some cases, a pre-wash step is carried out with showers to avoid accumulation of organic matter in the process water. Product is then usually immersed in a second tank in which the water may be treated with a sanitizer to prevent cross-contamination during washing (FAO, 2003) if permitted

by national regulations (FAO, 2003). Whenever disinfectants are used, the last stage before packaging should be the rinsing step. Rising water requires very low doses of disinfection agent to maintain its hygienic quality although potable water may be used for this last rinsing step (EFSA, 2014a). Application of a rinsing step using showers has been recommended (Gil et al., 2015). When sanitizer is not used companies rely on water is replacement at a sufficient frequency to prevent the build-up of organic material and prevent cross-contamination (Gil et al., 2009).

After washing, excess of water needs to be removed. The dewatering method used in most of the fresh-cut processing lines is centrifugation (Gil et al., 2015). The time and speed of centrifugation, or alternative dewatering systems, are key parameters to be adjusted for each product. To reduce tissue damage and consequent microbial deterioration in leafy greens that are too delicate to centrifugation, forced air or air-bed conveyors are recommended especially for leafy greens. These systems are widely used in Europe (Turatti, 2011).

The final operation in the processing of fresh-cut leafy greens takes place in the assembly and packaging room. Virtually all processed leafy greens are stored under refrigeration and modified atmosphere packaging to achieve the required commercial shelf life (Francis et al., 2012). In the assembly room, after inserting the correct amount of product into the packages, the packs are sealed. Polymeric films are used in an effort to maintain product quality, while extending shelf life (Gil & Selma, 2006).

Proper temperature control during storage and transportation is critical to maintain visual quality and crispiness and to delay microbial growth during the shelf life (Francis et al., 2012). Thus, the storage unit must maintain the fresh leafy vegetables at appropriate temperatures, which may differ between the types of product, packaging, and the expected

shelf life (AFDO, 2004; Wright, 2004). The recommended marketing temperature for RTE leafy greens is 7 ºC although operators apply lower temperatures to optimize their quality and shelf life (EFSA, 2014a). However, these products may occasionally be abused at higher temperatures (10 to 12 ºC) during storage in display cabinets (Oliveira et al., 2010).

2.3 Baby spinach production and processing in Spain

The focus of this thesis is on baby spinach, which is becoming an increasingly popular produce in Europe because it is known to be a healthy product that contains relatively high concentrations of bioactive compounds (Gil et al., 1999). In addition, it has the advantage of a short cultivation time and shelf-life, making baby spinach an excellent model crop.

Baby spinach (*S. oleracea* L.) belongs to the goosefoot family (*Chenopodiaceae*). The leaves are smooth, semi-savoy or savoy, depending on the cultivar. Baby spinach is harvested after a short growth period, no longer than 90 days, and sown at closer density than regular spinach, thus the leaves are smaller, hence the name. Spinach is a quick-maturing, cool-season vegetable crop. Seeds germinate at 2 to 30 ºC but 7 ºC to 24 ºC is optimal. Baby spinach grows from 5 to 30 ºC but growth is most rapid between 15 to 18 ºC (Lestrange et al., 1999).

In Spain, baby spinach production chain starts with the preparation of the soil by fertilizing at the beginning of the season. The preparation consists of application of fertilizer and ploughing the field to prepare the cultivation soil. Fertilizer can be either inorganic or organic; the most used fertilizer by Spanish leafy green growers is composted manure. It is an excellent source of nutrients that improves soil structure (Food Safety Agency UK, 2009). Animal manure consists of a mixture of cattle, bovine and poultry

manure. After application, a waiting time of at least 90 days is left before sowing (Spanish Companies information).

Baby spinach seeds are directly planted 1.2-2.0 cm deep, depending on the method of plating and soil conditions. A variety of soils is used for spinach production but sandy loam soils are preferred. Regarding irrigation, sprinkler method is used and applied generally every day with exception of rainy days. Spinach has a relatively shallow root system and thrives on frequent, short irrigations to maintain a uniformly moist soil for maximum leaf production (ANR, 2012). The crop cycle usually takes between 35-60 days, depending on the season (Spanish Companies information).

Picture 1.1. Cultivation field of baby spinach ready to harvest.

Once the crop has reached the desired commercial size (**Picture 1.1**), baby spinach is mechanically harvested. A machine with a front cutter bar is run on top of the plant beds (**Picture 1.2A**). The cutter bar clips the leaf and attached petiole of the plant. Mechanical

damage during harvest can become a serious problem, as plant injuries predispose produce to decay, increased water loss as well as increased respiratory rates which can accelerate deterioration (Kitinoja & Kader, 2002).

Picture 1.2. (A) Mechanical harvesting of baby spinach. **(B)** Detailed picture of the conveyor belt of a commercial harvesting machine.

After harvested, leaves are lifted by conveyor belts into bins on trailers and transported at refrigeration temperature to the processing plant (**Picture 1B**). Baby spinach must be cooled rapidly to prevent excessive weight loss and wilting as it has a large surface-to-weight ratio and a very high respiration rate. If fresh processing is delayed, baby spinach is typically vacuum cooled and stored for a short period (maximum two days) (Gil et al., 2006).

When harvested spinach reaches the processing plant, it is sorted and visually inspected to evaluate the incidence of defects (i.e. bruising, blemishes, freeze damage during transport, etc.) or insect infestation. This inspection is performed by trained workers in the manual operation area (**Figure 1.2**). After this manual operation, baby spinach is washed in chlorinated water tanks (one or normally two). The purpose of the washing step is pre-cooling, removing dirt, pesticide residues and cell exudates that may support microbial growth, and reduce the bacterial load (spoilage bacteria and pathogens) of the

produce (Gil et al., 2009). For leafy greens that float in the washing tank, as is the case of baby spinach, a washing system where high volumes of air are blown into the tank through pipes located just beneath the surface of the water is a currently used method. This creates a vigorous 'jacuzzi' effect, which causes produce to tumble around and creates the mechanical action needed for optimal cleaning (Gil et al., 2015). After washing, the produce is dried by centrifugation or forced-air drying and transported by means of conveyor belts to the packaging unit. As final step, baby spinach is packaged into different bagged spinach or mixed leafy green products (**Figure 1.2**). Baby spinach is generally packeged in polypropylene bags with a maximum storage time of about 10 days.

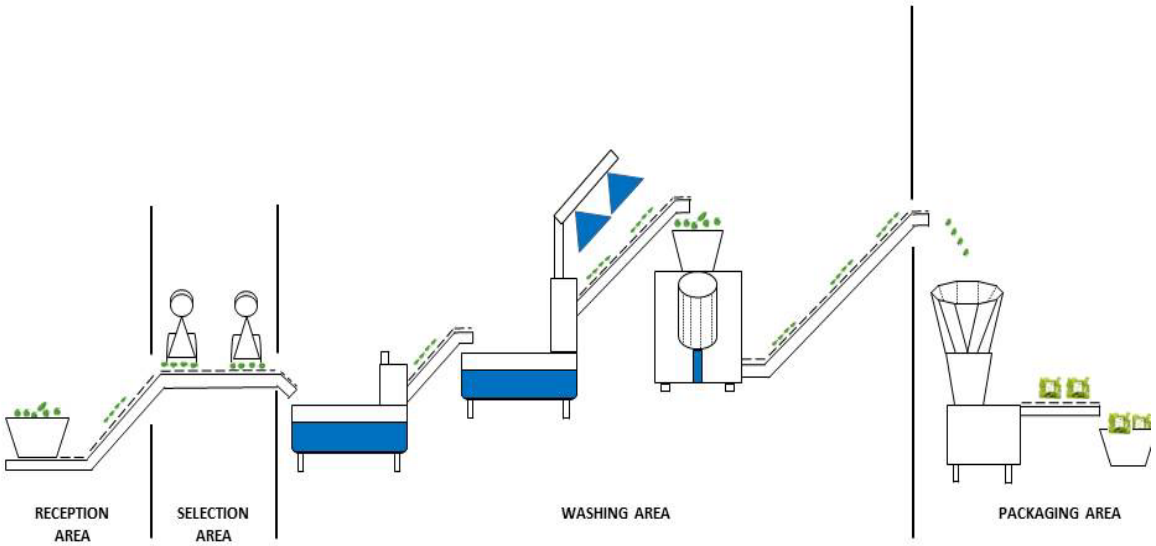

Figure 1.2. Scheme of leafy vegetables processing steps.

3. Leafy greens consumption in Europe and Spain

3.1 Recommendations and intake measurement

A healthy diet, including a daily consumption of 400-500 g of fruits and vegetables, is known to play an important role in human health and disease prevention (WHO, 2014). Several international organizations such as the World Health Organization encourage the

daily intake of at least 400 g of fruit and vegetables per day (excluding potatoes and other starchy tubers) for the prevention of chronic diseases such as heart disease, cancer, diabetes and obesity (WHO, 2014; Callejón et al., 2015). In Europe, recommendations vary between countries. In general, these agree with the WHO recommendation, but some countries recommend even higher amounts e.g. ≥ 600 g per day such as in Denmark (Yngve et al., 2005). All public health recommendations of fruit and vegetable intake should be flexible and adapted to local circumstances because of the wide variability of dietary patterns, food availability, food preferences and cultural considerations (FAO/WHO, 2004).

There are different ways to measure food consumption. Food diaries and dietary recalls (i.e. interviews and questionnaires) are ways to obtain information on what individuals eat. Household spend and average food supply based on national statistics may also be used to assess consumption (EUFIC, 2012). Different methods take into account different aspects and the exactitude varies between them. Hence, data obtained with different methods are not directly comparable. National Authorities have typically selected methods for their dietary surveys without international comparability in mind (WHO, 2006a). In general, there are several problems in the assessment of fruits and vegetables consumption in epidemiological studies and comparison across countries. Many studies are carried out in small groups with relatively homogeneous diets, and attenuation of differences because the measurement error cannot be discounted. A major issue when comparing results across populations is the validity and standardization of the instrument used to assess dietary intake (Agudo et al., 2002).

3.2 Importance and availability of consumption data

Recent food contamination events involving fresh vegetables have stressed the importance of the evaluation of the risk associated with their production chain through exposure assessment, a key step in risk assessment. In order to perform accurate risk assessment studies, information about food consumption is necessary to calculate exposure of the population to a certain food safety hazard (Hoelzer et al., 2012). These data must be recent and representative of the target population (EFSA, 2009b).

Several organizations such as the Food and Agriculture Organization of the United Nations (FAO) have provided data on food consumption based on agricultural data indicating the food supply patterns at national level. According to this, the vegetable supply in Europe has increased over the last four decades. There seems to be a trend towards lifestyle and dietary uniformity in Europe, with changes towards healthier diets in northern countries, while Mediterranean countries have moved towards more northern dietary patterns (Agudo et al., 2002). A north-south gradient has been observed; in Northern Europe the vegetable supply is lower than in Southern Europe. For example, in Finland, the average supply is 195 g per person per day, which corresponds to 71 kg per person per year, whereas Greece has an average supply of 756 g per person per day (276 kg per person per year) (Elmadfa et al., 2009).

Recently, Freshfel Europe has released the newest 12th edition of 'The latest fresh fruit and vegetable production, trade, supply & consumption monitor in the EU28' which covers the period from 2008 to 2013. This European organization identifies EU-wide trends relating to production, import and export and includes specific information on fresh fruit and vegetable net supply and consumption trends for the EU28. Findings from this latest edition show that the consumption of fresh fruit and vegetables in the EU28 stands at

341.81 g/capita/day in 2013. This is an increase of 5.6% compared with 2012 but a decrease of 1.9% compared with the average of the years 2008-2012. It means that consumption in the EU28 remains under the above-mentioned recommendation of the World Health Organization (Freshfel, 2014). In this report, the United Kingdom (UK) is the largest market of RTE fruits and vegetables, accounting for around a third of total. However, data related to consumption within the EU is controversial. A report presented by the organization "5 a day", the worldwide movement to promote Fruits & Vegetable consumption, stated that Spain consumes more fruit and vegetables than others European countries such as France, Italy, Germany or the UK. According to this study, Spanish people consume an average of 260 kg of fruit and 220 kg of vegetables per year.

In order to overcome the lack of reliable and comparable data on dietary intake, the EU research project EU menu was launched to harmonize consumption data among EU countries (EFSA, 2009b). However, since pre-standardization seemed impossible, the project was focused on post-harmonization of available intake data which is not always optimal for calculations and comparisons of dietary exposures among countries.

4. Safety considerations of leafy greens

Increasing production and consumption of leafy greens has been accompanied by a rise in the number of produce associated foodborne outbreaks worldwide (Mercamoglu Taban & Halkman, 2011; Callejón et al., 2015). Lately, leafy greens have been included in the five top ranking groups of food/pathogen combinations according to specific criteria in the EU and leafy greens eaten raw as salads were considered the highest priority group in terms of fresh produce safety from an EU perspective (EFSA, 2013).

4.1 Outbreaks related to leafy greens

Although contaminated samples of RTE foods are found occasionally in Europe, as documented by the Rapid Alert Systems for Food and Feed (EC, 2013), a number of previous laboratory surveys indicate that pathogenic organisms are uncommon in fresh products (Koseki et al., 2011; Althaus et al., 2012). When an outbreak occurs, tracing back to the source of the causing foodborne pathogen is challenging. This can be attributed to the relatively short shelf life of fresh produce, which is often discarded by the time an outbreak is identified. The lack of traceability of produce is a further contributing factor that makes identification of specific sources problematic (Warriner et al., 2009). However, it has been recently reported that nearly half of all foodborne outbreaks are attributed to contaminated produce, with leafy vegetables causing 22% of the total outbreaks in the United States (Painter et al., 2013). In fact, several of these outbreaks have involved multiple countries due to the large volume of international trade that occurs with this type of commodities (Callejón et al., 2015). The number of reported outbreaks (defined as the occurrence of two or more cases of similar illness resulting from the ingestion of a common food) represents only a portion of the real amount of outbreaks (Arendt et al., 2013). In the EU in 2009 and 2010, 4.4% and 10% respectively of the foodborne verified outbreaks were linked with the consumption of vegetables, fruits, berries, juices (and products thereof) (EFSA/ECDC, 2015). *Salmonella*, STEC and *E. coli* O157:H7 in particular have been related to the several fresh produce outbreaks over the last years (Callejón et al., 2015).

A recent study has reviewed the leafy vegetable-associated outbreaks reported to the Centres for Disease Control and Prevention (CDC) between 1973 and 2012. This study stated that during the study period, 606 leafy vegetable-associated outbreaks, with 20.003

associated illnesses, 1.030 hospitalizations, and 19 deaths were reported. On average, leafy vegetable-associated outbreaks were larger than those attributed to other food types (Herman et al., 2015). A compilation of publications from 2005 to 2015 that described investigations of outbreaks associated with leafy greens in US and Europe is presented in **Table 1.2**. As shown, different outbreaks implied a whole range of countries and different leafy green commodities were associated with high number of cases. Even though intense trace back research is carried out after the outbreaks, the contamination source is unlikely to be identified because trace back is complicated and time-consuming (Lienemann et al., 2011).

There are a number of possible reasons for the increase in produce-associated outbreaks over the last years such as an aging population that is more susceptible to foodborne illness; an increase in global trade; a more complex supply chain; improved surveillance and detection of foodborne illness; improvements in epidemiological investigation; and increasingly better methods to identify pathogens. Improved diagnostics and increased surveillance could lead to an increased identification but they would lead to an overall increase in reported foodborne outbreaks (FDA, 2009a; Tauxe et al., 1997; Jacxens et al., 2010).

Table 1.2. Foodborne outbreaks related to leafy greens occurred in the last 15 years.

Year	Causative agent	Affected Country	Cases	Implicated commodity	Reference
2000	*S. enterica Typhimurium*	Iceland, Netherlands	392	Lettuce	Crook et al., 2003
2000	*S. enterica Typhimurium*	UK	361	Lettuce	Horby et al., 2003
2001	*S. Newport*	UK	9	RTE salad	Fisher & O'Brien, 2001
2003	*E. coli* 0175	US	57	Mixed salad	CDHS, 2005
2004	*S. enterica Thompson*	Norway	20	Ruccola	Nygard et al., 2008
2004	*S. enterica Newport*	UK	368	Lettuce	Gillespie, 2004
2004	*S. enterica* Braenderup	UK	40	Iceberg lettuce	Gajraj et al., 2012
2005	*S. enterica Typhimurium* DT104	Finland	56	Lettuce	Takkinen et al., 2005
2005	*S. enterica Typhimurium* DT104	UK	96	Iceberg lettuce	Little & Gillespie, 2008
2005	*E. coli* O157	Sweden	135	Iceberg lettuce	Söderstrom et al., 2008
2006	*E. coli* O157	US	205	Pre-packaged spinach	Jay et al., 2007; CDC, 2006
2006	*E. coli* O157	US	200	Bagged spinach	Gelting et al., 2011
2006	NoV (GII.7)	Austria	182	Bagged salad	Schmid et al., 2007
2007	*E. coli* O157	Netherlands, Iceland	50	RTE salad	Friesema et al., 2008
2007	NoV (GII.4)	UK	34	Mixed salad	Showell et al., 2007
2007	NoV (GII.6)	UK	79	Salad	Vivancos et al., 2009
2007	NoV (GII.3)	Sweden	413	Salad buffet vegetables	Zomer et al., 2010
2007	NoV (GII.4)	Japan	23	Salad vegetables	Oogane et al., 2008
2009	NoV (GII.4)	Germany	27	Salad	Wadl et al., 2010
2010	Enterotoxigenic *E. coli*	Denmark, Norway	260	Lollo biondo lettuce	Ethelberg et al., 2010
2010	*E. coli* O145	US	26	Romaine lettuce	CDC, 2010a
2013	NoV (GI and GII)	Denmark	260	Lettuce	Ethelberg et al., 2010
2011	*E. coli* O157	US	58	Romaine lettuce	CDC, 2012a; Slayton et al., 2013
2012	*E. coli* O157	US	33	Spinach and Salad mix	CDC, 2012b
2013	*E. coli* O157	UK	19	Watercress	Jenkins et al., 2015
2013	Enterotoxigenic *E. coli*	Sweden	19	Fresh salad	Eldestein at al., 2014
2013	*E. coli* O157	Mexico	59	Lettuce	Foodborne outbreak database, 2013
2015	*E. coli* O157	Canada	13	Leafy greens	PHAC, 2015
2015	*E. coli* O157	UK	38	RTE Salad	Food Safety News, 2015

4.2 Pathogenic microorganisms related to leafy greens

A wide spectrum of pathogens and food vehicles has been documented in produce-associated outbreaks (Berger et al., 2010). A literature survey of human pathogens listed more than 1400 potential food-contaminating species, 58% of which are known to be zoonotic i.e. pathogens that can naturally be transmitted between vertebrate animals and humans. Among these 1400 pathogens, 538 (38%) were bacterial species that are generally characterized by broad host range and seemed to exploit almost any change in human ecology that provides new opportunities for transmission (Woolhouse & Gowtage-Sequeria, 2005). Survey studies designed to investigate the presence of enteric pathogens in leafy greens have shown that contamination with pathogens occurs infrequently and at low levels (Mukherjee et al., 2004, 2006; Berger et al., 2010; Koseki et al., 2011). However, data from literature are sometimes inconsistent about the prevalence of these pathogens due to significant differences among studies related to the size and place of sampling, fresh produce type, seasonality and analytical methods (FAO/WHO, 2008). The pathogens most frequently associated with leafy green vegetables and on which this thesis focuses on are: *L. monocytogenes*, *Salmonella* spp. and pathogenic *E. coli* strains, especially STEC, including O157:H7 (Scallan et al., 2011; Pérez-Rodriguez et al., 2014).

4.2.1 Listeria monocytogenes

L. monocytogenes causes human and animal disease. This is the only *Listeria* species that represents a human public health concern being, for that reason, the most important species of this genus (Milillo et al., 2012). This microorganism is widespread in the environment and commonly occurs in sewage, sewage sludge, silage, soil, straw, hay, grass, vegetable materials, animal feed, drains, machinery, and surface waters (Orsi et al., 2011; Ferreira et al., 2014). Furthermore, this pathogen has been isolated from the

faeces of poultry, wild birds and a number of wildlife species, including deer, moose, otters and raccoons (Hellström et al., 2008; Lapen et al., 2007). Infection with *L. monocytogenes* can cause several different forms of listeriosis in pregnant women, neonates and the immunosuppressed and elderly individuals. Nevertheless, healthy people can also be infected by this pathogen (Milillo et al., 2012). Symptoms of listeriosis include diarrhoea, fever, and muscle aches. More serious complications associated with human listeriosis include stillbirth, septicemia and infections of the central nervous system (meningoencephalitis, encephalitis) (Rocourt et al., 2000; Lomonaco et al., 2015).

Data from the Foodborne Diseases Active Surveillance Network (FoodNet) show that, even though human listeriosis is less common that many other foodborne diseases, it is by far the most severe (Pouillot et al., 2012). Exposure to moderately high doses is necessary for causing infection and disease. Additionally, variations in virulence of strains together with differences in host susceptibility may also contribute to the fact that exposure to *L. monocytogenes* from contaminated foods rarely appears to cause disease (Notermans & Hoornstra, 2000).

In several countries, criteria or recommendations for tolerable levels of *L. monocytogenes* in RTE foods have been established. Some countries, such as the US, require absence of *L. monocytogenes* in 25 g of foods (zero tolerance) (FDA, 2008). Food safety criteria in Spain for fresh-cut fruits and vegetables are regulated by the Commission Regulation EU Nº 1441/2007 (OJEU L322/12-29, 7 December 2007) as a follow up of Regulation EU Nº 2073/2005 (OJEU L338/1-26, 22 December 2005). In ready-to-eat foods able to support growth of *L. monocytogenes*, absence of *L. monocytogenes* is demanded in 25 g before the food has left the immediate control of

the producing food business operator and <100 CFU/g in products placed on the market during their shelf life (**Table 1.3**).

Table 1.3. Food safety criteria for *L. monocytogenes* and *Salmonella* in vegetables and fruits.

Food category	Microorganism	Sampling plan		Limits		Stage where criteria applies
		n	***c***	***m***	***M***	
RTE foods able to support microorganism growth	*L. monocytogenes*	5	10	100 CFU/g		Products placed on the market during their shelf life
		5	10	Absence in 25 g		Before the food has left the immediate control of the food business operator, who has produced it
RTE fruit and vegetables	*Salmonella spp.*	5	0	Absence in 25 g		Products placed on the market during their shelf life

n = number of units comprising the sample; c = number of sample units giving values between m and M.

In recent years, while cases of listeriosis involving fresh produce are few, several listeriosis outbreaks have been linked to the consumption of fresh or processed produce (Hoelzer et al., 2012). In the EU during 2007 to 2011, analytical epidemiology identified a significant risk amongst sporadic cases in England and Wales with consumption of pre-packed mixed salad (Little &Gillespie, 2008; Little et al., 2010).

Additionally, several studies have found this microorganism in RTE salads in different countries such as US (Gombas et al., 2003), Brazil (Porto & Eiroa, 2001; Fröder et al., 2007), Chile (Cordano & Jaquet, 2009), UK (Sagoo, et al., 2003; Little et al., 2007), Spain (Cabedo et al., 2008; Pérez-Rodriguez et al., 2014) and Italy (Losio et al., 2015). Crépet et al. (2007) analysed 165 studies and reported that prevalence of *L. monocytogenes* on salad vegetables is usually lower than 5%. This paper also showed that surveys conducted after 2000 reported lower instances of *L. monocytogenes* isolation. This suggests that increased knowledge of pathogen behaviour in the food

processing environment and more effective sanitization procedures have led to improved product control (Heaton & Jones, 2008). One major determinant of the listeriosis risk is the ability of a food to support growth of *L. monocytogenes* during storage (Hoelzer et al., 2012). Some studies have demonstrated that *L. monocytogenes* can grow on a variety of vegetables even at refrigeration temperatures (Francis and O'Beirne, 1997; Brackett, 1999; Jacxsens et al., 2001; Carrasco et al., 2008).

4.2.2 *Salmonella* spp.

Salmonella species are probably the most well known bacterial foodborne pathogens. They are Gram negative, facultative anaerobic, rod-shaped, non-spore forming, motile bacteria which belong to the family of *Enterobacteriaceae* (Ellermeier & Slauch, 2006). The microorganisms are mesophilic, with optimum growth temperatures of 35-43 ºC. They can metabolize a wide variety of organic substrates by both respiratory and fermentative pathways. The genus *Salmonella* encompasses a large taxonomic group with around 2500 recognized serovars (Fatica & Schneider, 2011; Ellermeier & Slauch, 2006). They are classified according to biochemical characteristics and the immunoreactivity of two surface structures, the O and H antigens. According to the CDC system, the genus *Salmonella* contains two species (*S. enterica* and *S. bongori*), each of which contains multiple serotypes (CDC, 2015b). The Centres for Disease Control and Prevention (CDC) estimated that 95% of *Salmonella*-based infections originate from foodborne sources (CDC, 2015b). Several *Salmonella* serovars have been associated with both animal- and produce-based outbreaks; in particular *S. enterica* serovar Enteritidis and *S. enterica* serovar Thyphimurium have been associated with more than half of the reported salmonellosis cases (Lan et al., 2009).

The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals. This pathogen is usually transmitted by ingestion of food or

water contaminated by infected faeces (EFSA, 2013). *S. enterica* serotypes Enteritidis and Typhimurium are encountered most frequently worldwide and are the two most important serotypes for salmonellosis transmitted from animals to humans (EFSA, 2010a). The Centres for Disease Control and Prevention estimated that 95% of *Salmonella*-based infections originate from foodborne sources (CDC, 2015b).

In relation to the clinical relevance of this pathogen, the two major clinical syndromes caused by *Salmonella* infection in humans are enteric or typhoid fever and colitis/diarrheal disease. Enteric fever is a systemic invasive illness with clinical manifestations of fever, headache, abdominal pain, and transient diarrhoea or constipation, and infection that can produce fatal respiratory, hepatic, spleen, and/or neurological damage. Without treatment, the mortality is 10 to 20%, decreasing to 1% among patients treated with the appropriate antibiotics (Fábrega & Vila, 2013).

On the other hand, there are many non-typhoidal *Salmonella* (NTS) strains that cause diarrheal disease in humans and can, in addition, infect a wide range of animal hosts (Ohl & Miller, 2001; Gordon, 2008). Gastroenteritis is by far the most common manifestation of disease caused by *Salmonella*. The incubation period is typically 6- 48 h and it is followed by fever, abdominal pain, nausea, and sometimes vomiting. However, in some patients, particularly in the very young and elderly adults, the infection may be more serious and the associated dehydration can be life threatening (Coburn et al., 2007). It is estimated that more than 10^5 cells are required to initiate an infection (LaRock et al., 2015). However, in some outbreaks the infectious dose was reported to be as low as 10 to 100 cells. The exact amount needed for infection depends on the type of food, type of strain, the physiological state of bacteria and characteristics of the host (Fábrega & Vila, 2013). The establishment of a human *Salmonella* infection depends on the ability to survive the environment outside the host, the ability to survive

the gastric acid of the human stomach and the ability of the pathogen to attach and invade intestinal cells (Franz & van Bruggen, 2008). In relation to survival, *Salmonella* spp. has been proven to survive in the phyllosphere despite the harsh and variable conditions (Fatica & Schneider, 2011). In fact, on the crop surface the bacteria are exposed to high doses of UV light, poor nutrients, aerobic environment and inconstant temperature (Whipps et al., 2008). The microbiological criterion for *Salmonella* spp. in fresh-cut fruits and vegetables is absence of *Salmonella* in 25 g of foods (zero tolerance, Commission Regulation EU Nº 1441/2007, OJEU L322/12-29, 7 December 2007) (**Table 1.3**). *Salmonella* spp. has been detected regularly on surveys conducted on leafy green (whole crops or fresh-cut) either sampled at farm level, fresh-cut processing companies or in distribution or retail establishments in different countries worldwide such as at farm level in the US (Mukherjee et al., 2004), in a processing company in The Netherlands (Pielaat et al., 2008) and at retail level in Spain (Abadias et al., 2008), Mexico (Quiroz-Santiago et al., 2009) and Canada (Arthur et al., 2007). Conversely, in a high number of studies this pathogen has not been detected on leafy greens at any level in different countries within EU such as Belgium (Holvoet et al., 2014a), Spain (Oliviera et al., 2010) and Norway (Jonhannessen et al., 2002; Loncarevic et al., 2005) as well as Singapore (Seow et al., 2012). Thus, the overall prevalence on leafy greens is assumed to be <1% (EFSA, 2013). Despite the low prevalence and the microbiological criteria of absence in 25 g, the number of produce outbreaks associated with *Salmonella* spp. has increased over the last years (Fatika & Schneider, 2011). Previous to 1990, most cases of salmonellosis were attributed to contaminated poultry and poultry products (Tauxe et al., 1997). In 2002–2003, 31 *Salmonella* spp. outbreaks of produce origin were reported in comparison to the 29 reported in poultry-related foods (CDC, 2008a). In 2004, Sivapalasingam et al. (2004) reported that *Salmonella* spp. was implicated in 18% of the lettuce associated outbreaks and 10% of the produce-related

Salmonella outbreaks were associated with lettuce in US between 1973 and 1997. In Europe, between 2000 and 2005, several outbreaks were linked to *Salmonella* and lettuce (Anonymous, 2003a; Crook et al., 2003; Horby et al., 2003; Takkinen, 2005). More recently, *Salmonella* spp. has been associated with a variety of produce outbreaks (Sivapalasingam et al., 2004; CDC, 2008b, 2013b) (**Table 1.2**). A recent study using a risk-ranking model situated the combination of *Salmonella* spp. and leafy greens as the food/pathogen combination most often linked with foods of non-animal origin between 2007 and 2011 in the EU (Da Silva Felício et al., 2015).

Regarding the most common serotypes, between 2004 and 2012, *Salmonella Enteritidis* was the most common serotype in the EU, associated primarily with salad, followed by *Salmonella* Newport, which was mostly related to the consumption of lettuce according to EFSA Summary Reports (Callejón et al., 2015). In 2004, an outbreak of *Salmonella* Thompson infections was linked to imported lettuce from Italy (Nygard et al., 2008). Several years later, iceberg lettuce was suggested as possible source of a nationwide outbreak caused by two *Salmonella* serotypes, Newport and Reading, in Finland (Lienemann et al., 2011). Recently, the serotype *S. Braenderup* was first reported to cause an outbreak due to lettuce from Spain to the UK (Gajraj et al., 2012).

4.2.3 *E. coli* pathogenic strains

Escherichia coli are Gram-negative, non-sporulating straight rod, facultative anaerobic, oxidase negative, catalase positive bacteria belonging to the family *Enterobacteriaceae* (Rajwar et al., 2015). This microorganism is mesophilic, with optimum temperatures for growth of 35-40 ºC. It is considered part of the normal microbiota of the intestinal tract of humans and most other warm-blooded animals. Hence, it is generally present in faeces. Most strains of *E. coli* are harmless, but a small

proportion has evolved into pathogens that can cause serious clinical symptoms in humans (Kaper et al., 2004; Clements et al., 2012).

There are several types of *E. coli* strains that may cause gastrointestinal illness in humans. The pathogenic *E. coli* uses a multi-step scheme of pathogenesis, which consists of colonization of the mucosal site, evasion of host defences and, multiplication and host damage (Kaper et al., 2004). Pathogenic *E. coli* strains can be divided into six groups or pathotypes. (Clements et al., 2012) that present different virulence factors, clinical symptoms and serotypes (EFSA, 2013).

- **Shiga-toxin producing *E. coli* (STEC) or verocytotoxin producing *E. coli* (VTEC)**

Amongst *E. coli* capable of causing intestinal disease, this group, also known as STEC are strongly associated with the most severe forms of infection (EFSA, 2013). This group has a definite zoonotic origin with cattle being recognized as the major reservoir while humans are unlike (Caprioli et al., 2005; Clements et al., 2012). STEC colonization in adult ruminants is asymptomatic (Wray et al., 2000). This group has been associated with outbreaks worldwide representing a serious public health concern (Nguyen & Sperandio, 2012). In 2013, the number of confirmed cases of STEC infections in the EU was 6.043. The EU notification rate was 1.59 cases per 100.000 inhabitants, which was 5.9 % higher than in 2012 (EFSA/ECDC, 2015). Over 380 different VTEC serotypes have been isolated from humans and animals, but only a small number of serotypes are linked to human disease. Serotype O157:H7 is the major source of *E. coli* food poisoning outbreaks in the US (Karmali et al., 2010). It was first identified in 1982 as the causative agent of bloody diarrhoea and haemolytic uremic syndrome in humans. This serotype has been recognized as emerging foodborne pathogen of great concern (IOM, 2012) associated with the consumption of undercooked beef (Riley et al., 1983). Moreover, *E. coli* O157:H7 is generally

considered to be more virulent than other STEC because of the severity of illnesses caused in spite of the apparent low infective dose (Bach et al., 2002; Vanselow et al., 2005). In fact, it is considered one of the most serious known foodborne pathogens although the reason for this is unclear (Blanco et al., 2004). Characteristics of *E. coli* serotype O157:H7 infection include abdominal cramps and bloody diarrhoea, as well as the life-threatening complication of haemolytic uremic syndrome (HUS) (Nguyen, 2012). Currently, no treatment is available for STEC infections and the use of conventional antibiotics exacerbates Shiga toxin-mediated cytotoxicity (Goldwater & Bettelheim, 2012). One of the key virulence factors of STEC is the ability to produce Shiga-toxins (Stx) which consists of two types: Stx1 and Stx2, which play a crucial role in causing HC and HUS (Kaper et al., 2004). The high virulence of STEC strains is not only determined by genes coding for toxins and adherence factors but also by its ability to survive environmental stresses (Smith et al., 2014). Their capacity to colonize the human gut is for a large part due to their resistance to low pH levels like encountered in the human stomach, resulting in a relatively low infectious dose, which has been estimated to be occasionally as low as 50-100 cells (Smith et al., 2014).

STEC infections occur worldwide but they are most common in the US and Canada (Callejón et al., 2015). *E. coli* O157:H7 is the most prevalent strain (Sivapalasingam et al., 2004; Heaton & Jones, 2008; Berger et al., 2010; Callejón et al., 2015). Leafy greens have been associated to several outbreaks of *E. coli* O157:H7 (Slayton et al., 2013; Ethelberg et al., 2010; Friesema et al., 2007, 2008; Söderstrom et al. 2008) (**Table 1.2**). In 2006, a major outbreak of foodborne illness associated with the consumption of spinach tainted with EHEC occurred in the US. This episode was linked to contamination of a spinach field by STEC-infected wild pigs roaming in the Salinas Valley in California (Cooley et al., 2007; FDA, 2007). In 2011, a multistate outbreak

was reported, with 58 cases of food poisoning been linked to the presence of *E. coli* O157:H7 in romaine lettuce in five different cities in the US (Slayton et al., 2013).

- **Enteropathogenic *E. coli* (EPEC)**

The first strain of *E. coli* generally accepted to cause diarrhogenic outbreaks in the developed world was EPEC (Neter et al., 1955). Its incidence has declined and EPEC outbreaks are now rare in developed countries (Clements et al., 2012). However, it does remain an important cause of infant diarrhoea in the developing world (Ochoa et al., 2008; Hu & Torres, 2015). EPEC induce a distinctive histopathology known as the attaching and effacing (A/E) lesion, which is characterized by the intimate attachment of bacteria to the epithelial surface and effacement of host enterocyte microvilli. There are three stages in EPEC pathogenesis: (i) initial adherence to the host cell, (ii) production and translocation of bacterial proteins through a needle complex via a type III secretory system, and (iii) intimate bacterial attachment with pedestal formation (Chen & Frankel, 2005). EPEC is a significant cause of infectious diarrhoea that is often accompanied by fever, vomiting, and dehydration in children under 2 year old (Kaper et al., 2004). Acute diarrhoea is the most likely result of EPEC (Naguyen & Sperandio, 2012). In comparison with the infection caused by other diarrheal pathogens such as adenovirus, rotavirus, *Campylobacter* and *Salmonella*, the infection with EPEC is more likely to lead to development of persistent diarrhoea and hospitalization (Naguyen & Sperandio, 2012). There are a number of other EPEC virulence proteins (invasion and effector proteins and toxins) that might be important for the clinical manifestations of intestinal infection in children. However, the association and relevance of these proteins to

the development of prolonged diarrhoea and other clinical manifestations is not well established (Ochoa et al., 2008).

- **Enteroaggregative *E. coli* (EAEC)**

The Enteroaggregative *E. coli* (EAEC) group is a large, diverse group of diarrhoeagenic *E. coli*. EAEC was first described in 1987 by Nataro et al. (1987) in a study examining different patterns of adherence of *E. coli* strains to HEp-2 cells in culture (Dallman et al., 2014). However, its pathogenicity and clinical relevance are still controversial (Jensen et al., 2014). The aggregative adherence (AA) pattern is defined as the binding of bacteria to epithelial cells in a stacked-brick manner. Although EAEC has been associated with diarrhoea in studies conducted in both developing and industrialized countries, it has been difficult to determine the specific mechanisms of EAEC pathogenicity, which has made difficult the assessments of the clinical relevance of this microorganism (Jensen et al., 2014). Virulence gene content associated with EAEC is highly variable between different strains, as illustrated in studies aimed at genotyping EAEC from a variety of clinical sources, healthy control groups and outbreaks (Okele & Nataro, 2001; Jenkins et al., 2003). This pathotype is the second most common cause of travellers' diarrhoea after ETEC (Shah et al., 2009). Its prevalence in endemic and epidemic disease is becoming well recognized. It also causes persistent diarrhoea in children in developing countries (Sarantuya et al., 2004) and has been implicated as an important enteric pathogen affecting AIDS patients (Clements et al., 2012). There has not been described an animal reservoir for EAEC, suggesting that it is persisting in the human population (Beutin & Martin, 2012; Oundo et al., 2008). The transmission of EAEC is often from foodborne or through contaminated water, and as such, it is believed to be transmitted by the faecal-oral route (Jensen et al., 2014).

This serotype could be now considered and emerging pathotype of Enteric *E. coli* that requires continued detection of this population of hybrid EAEC/EHEC strains since the 2011 German *E. coli* foodborne outbreak which was caused by an EAEC strain (O104:H4) that acquired typical STEC pathotypes, most notably Stx production (Clements et al., 2012).

- **Enterotoxigenic *E. coli* (ETEC)**

Enterotoxigenic *E. coli* (ETEC) are a diverse group of pathogens that have in common the ability to colonize the small intestine, where they produce and deliver plasmid-encoded heat-labile (LT) and/or heat-stable (ST) enterotoxins. Collectively, these organisms cause hundreds of millions of cases of diarrheal disease each year, particularly in developing countries. ETEC are responsible for an estimated 300.000-500.000 death per year in children under five years old (WHO, 2006b). ETEC has been established as the most common cause of diarrhoea in travellers as well as in young children in resource-limited regions of the world (Isidean et al., 2011). ETEC infections are classically associated with acute watery diarrhoea. Like clinical cholera, these infections can range from mildly symptomatic to severe profuse cholera-like watery diarrhoea leading to rapid dehydration and prostration within a few hours (Fleckenstein et al., 2010). In addition to diarrhoea, other signs and symptoms including headache, fever, nausea and vomiting are often reported, and some patients may have prolonged diarrheal illness lasting a week or more (Isidean et al., 2011).

ETEC infections are transmitted through the faecal-oral route. Exposure to ETEC is usually from contaminated food and drinking water (Croxen et al., 2013). Some examples of high-risk foods contaminated with etiological agents for traveller's diarrhoea include food that is left at room temperature, table-top sauces, certain fruits, and food from street vendors (Hill & Beeching, 2010). Additionally, surface water in

developing regions can also contain these organisms and may serve as an important source of infection during contact with this water (Begum et al., 2005). Foods can be contaminated by infected food handlers (Jain et al., 2008), by asymptomatic carriers (Taneja et al., 2010), or when vegetable crops are irrigated with untreated water (Castro-Rosas et al., 2012). Although occurrence of outbreak associated with ETEC and leafy greens are rare, in 2010 an outbreak related with *Lollo biondo* lettuce contaminated with *E. coli* O6:K15:H16 serotype was reported to affect 246 people (Ethelberg et al., 2010).

- **Enteroinvasive *E. coli* (EIEC)**

This pathotype and *Shigella* are very similar because they share the same virulence mechanisms and disease symptoms although they can be distinguished by minor biochemical tests (Clements et al., 2012). EIEC was first described 50 years latter (Lan et al., 2004). Strains of EIEC and *Shigella* appear to have evolved independently and EIEC strains seen to be intermediates between *E. coli* and *Shigella* (Clements et al., 2012). EIEC comprises 21 major serotypes, which are typically defined by their O-antigen pattern, with a few exceptions that also present H antigens (Scheutz & Strockbine, 2005). Conventional host-to-host transmission of EIEC and *Shigella* is mediated via the faecal-oral route mainly through contaminated water and food or direct person-to- person spread (Lampel, 2012). EIEC is underrepresented in epidemiological surveys due to its less severe clinical manifestations. In addition, EIEC strains with very close biochemical, genetic and pathogenic similarity to *Shigella* might be misclassified, whereas EIEC strains with commensal *E. coli* characteristics, such as lactose fermentation, might remain undetected in respective surveys (Croxen et al., 2013). Thus, no EIEC episodes have been reported in recent health-related government surveillance programs led in the US, Canada, Europe and Australia (CDC, 2012; NESP, 2010; ECDC, 2011; OzFoodnet, 2012).

- **Diffusely adherent *E. coli* (DAEC)**

The diffusely adherent *E. coli* (DAEC) pathotype describes diarrheagenic *E. coli* strains that attach to cells but do not fall into classical patterns of adherence, such as localized or A/E (Croxen et al., 2013). They have now emerged as a unique group and are considered distinct from other pathotypes, but because of difficulties in classification and identification, the designation of DAEC as a distinct enteric *E. coli* pathotype requires further epidemiological studies (Clements et al., 2012).

The importance of DAEC to enteric disease remains uncertain and detection methods for diarrheagenic strains of DAEC are still being developed. Currently, there is no universal method to detect strains that are responsible for diarrhoea in a clinical setting. Thus, the epidemiology of diarrheagenic DAEC remains unclear (Croxen et al., 2013). Some studies suggest DAEC may be an important contributor to diarrheagenic disease in children. However, the problem of cross-reactivity of one of the standard detection probes raises questions about this pathotype (Snelling et al., 2009). A correlation with disease may occur in specific age demographics although further epidemiological studies are required if DAEC is to remain a distinct enteric *E. coli* pathotype (Croxen et al., 2013).

- **Adherent–invasive *E. coli* (AIEC)**

Adherent–invasive *E. coli* have recently emerged as an exciting potential etiological agent of Crohn's disease. AIEC are distinguished from commensal strains of *E. coli* through their ability to adhere to and invade epithelial cells and replicate in macrophages (Smith et al., 2013). The AIEC pathotype does not express common virulence factors found in various other pathogenic *E. coli* strains and the genetic basis for its pro-inflammatory and invasive phenotype is not fully understood (Nash et al., 2010).

4.2.4 *Campylobacter* spp.

Thermotolerant *Campylobacter* spp. (particularly *Campylobacter jejuni* and *Campylobacter coli*) commonly occurs in the faeces of wild birds, broiler chickens and sometimes other animals including also rodents and insects (Whiley et al., 2013). Contamination results from direct or indirect contact with avian or mammalian faeces and survival of *C. jejuni* in water or on various types of fresh produce may occur which is sufficient to pose a risk to the consumers (EFSA, 2013). *C. jejuni* was reported as the leading cause of bacterial foodborne gastroenteritis throughout the world, usually associated with broiler meat (Friedman et al., 2000; EFSA/EDCD, 2015). The organism is microaerophilic and can only grow at temperatures above 30 °C (Park, 2002). The required growth conditions are met in the gastro-intestinal tract of warm-blooded animals, including birds, the main reservoir for *C. jejuni*. Transmission to humans is considered to occur mainly via foods of animal origin, especially poultry and raw milk, and contaminated drinking water (Hynds et al., 2014; Braeye et al., 2015). However transmission of *C. jejuni* through contaminated fresh produce of non-animal origin, i.e. raw vegetables and fruit, might also be of significant importance (Verhoeff-Bakkenes et al., 2011; Whiley et al., 2013).

The prevalence of *Campylobacter* in leafy greens has been assessed in several studies (Chai et al., 2007; Holvoet et al., 2014a; Pielaat et al., 2014) and also in water used for fresh produce irrigation (Gu et al., 2013; Holvoet et a., 2014a). A microbiological survey of RTE leafy green marketed in Italy confirmed the presence of this microorganism in 4 out of 1160 RTE samples (Losio et al., 2015). Recently, Holvoet et al. (2014a) found a prevalence of 9% (8/80) in lettuce samples taken at a farm level in Belgium. Lower prevalence was found in two different studies carried out in the Netherlands in 2011where Verhoeff-Bakkenes et al. (2011) reported that only 2

out of 562 (0.003%) of leafy green samples (1 endive and 1 watercress) were positive for *C. jejuni* and recently, Wijnands et al. (2014) reported a prevalence ranging from 0.83% in endive to 2.7% in oak tree lettuce. However, other studies did not found *Campylobacter* in 128 lettuce samples from farmer markets in Canada (Bohaychuk et al., 2009) or in 237 RTE salads from retail establishments in Spain (Abadias et al., 2008).

Leafy greens associated with *Campylobacter* spp. disease are rare, or rarely reported. Analytical epidemiological studies amongst sporadic cases of *Campylobacter* disease identified consumption of lettuce in Ireland (Danis et al., 2009) and salad products in Wales (Evans et al., 2003). The most recent outbreak reported was associated with consumption of raw peas (Gardner et al., 2011).

4.2.5 Noroviruses

Noroviruses (NoV) are a group of related viruses that can cause gastroenteritis which is inflammation of the stomach and intestines. This leads to cramping, nausea, vomiting, and diarrhoea (CDC, 2014). NoV can be divided into distinct genogroups based on phylogenetic analyses of the capsid protein. To date, five NoV genogroups (G) have been recognized (GI-GV). Viruses of GI, GII and GIV are known to infect humans (EFSA, 2011a). Human noroviruses (HuNoVs) are a major cause of gastroenteritis throughout the world, and the most common cause of foodborne illness in the US and Europe (CDC, 2013a; EFSA, 2009a). Several factors enhance the transmissibility of norovirus, including the small inoculum required to produce infection (<100 viral particles), prolonged viral shedding, and its ability to survive in the environment (Robilotti et al., 2015). NoV can be introduced on fresh produce by contact with contaminated faecal material, which can occur at any point during food production, harvest, processing and preparation. At the pre-harvest level, contact with polluted

irrigation water (Cheong et al., 2009) or organic fertilizer is possible (Wei et al., 2010). During harvest, in particular during handpicking of crops, an infected person could transmit the virus and at the post-harvest level the produce could be contaminated by contact with polluted process water or during food preparation by infected food handlers (Baert et al., 2008; Kokkinos et al., 2012). Furthermore, NoV can be expected to persist up to several weeks on vegetable crops which have been in contact with contaminated sewage or irrigation water, and, on fresh produce under conditions commonly used for storage in households, at least as long as the time generally taken between purchase and consumption (Esseili et al., 2015). The overall message is that NoV, once it has contaminated a foodstuff at source, it can remain infectious long enough for consumption of that foodstuff to constitute a risk to the consumer (EFSA, 2011a).

Although NoV presence has been linked to leafy greens, until recently, no data was available about the prevalence of NoV on fresh produce (Pérez-Rodríguez et al., 2014). Nevertheless, several NoV related outbreaks linked to leafy greens have been reported in Denmark (Ethelberg et al., 2010), Finland (Makary et al., 2009), Germany (Wadl et al., 2010), Austria (Schmid et al., 2007) and UK (Gallimore et al., 2005). In a recently published study which reviews the outbreaks related to fresh produce between 2004 and 2012, NoV have been reported as the leading cause of foodborne illness with 26 outbreaks related to leafy greens in EU (Callejón et al., 2015). This is likely to be a much larger contributor to produce-associated outbreaks than previously reported due to improvement in diagnostics (Radin & D'Souza, 2011).

In a study conducted in Canada, Belgium and France, NoV genomes were frequently detected in leafy greens (Baert et al., 2011). However, sequence confirmation was not successful for the majority of the samples tested. In Canada, between April and November 2009, 275 samples of packaged leafy greens were evaluated for the presence

of NoV and rotavirus and 148 samples (54%) were positive for NoV (Mattison et al., 2009). Recent European data can be found in the study by Kokkinos et al. (2012) in lettuce sold at retail in three European countries (Greece, Serbia and Poland), where 2/149 and 1/126 samples were positive for NoV GI and GII respectively. In Spain, NoV GII was recently found in 2/30 samples of RTE lettuce (Pérez-Rodríguez et al., 2014). Furthermore, adenovirus and NoV contamination was found in one sample of spinach at farm level in South Korea (Cheong et al., 2009) and in one sample of spinach at point-of-sale in Turkey (Yilmaz et al, 2011).

5. Risk factors

Leafy greens can become contaminated with foodborne pathogens at various stages of their production: during production, harvesting, processing, distribution and preparation at home. Production practices, growth conditions and the location of the edible part during growth in combination with intrinsic, extrinsic, harvesting and processing factors affect the microbial status of leafy greens at the time of consumption (EFSA, 2014a). Therefore, prevention is the most important measure and potential sources of contamination from the cultivation field to the table should be identified. Understanding the ecology of foodborne pathogens in the produce production and processing environment are critical for minimizing the risk of produce contamination (Gil et al., 2015). The following sections are intended to describe potential risk factors for contamination of leafy greens at pre-harvest and post-harvest levels.

5.1 Sources and factors affecting the likelihood of pre-harvest contamination

There are several sources of contamination and factors affecting contamination at pre-harvest level (**Figure 1.3**). The identification of factors and practices associated with an increased risk of produce contamination is still challenging because environmental factors and management practices often interact and differ within and between farms (Weller et al., 2015). Prior to harvest, produce can come into contact with both pathogenic and non-pathogenic microorganism in the field through different ways, such as irrigation water (Holvoet el al., 2014a; Allende & Monaghan, 2015), soil (Selma et al., 2012; Strawn et al., 2013b), manure applied as fertilizer (Franz et al., 2008a; Wilkes et al., 2011) and harvesting equipment and workers (FDA, 2009a). Additionally, many more factors, such as solar radiation (Solic et al., 1992; Jacobs et al., 2005), land topography (Strawn et al., 2013a), agricultural practices (Park et al., 2014; Wilkes et al., 2011) and climatic conditions (Ailes et al., 2008; Holvoet et al., 2014a; Park et al., 2014) may further influence the proliferation and survival of microorganisms on leafy greens. Thus, profound knowledge of the contamination sources and pathways for introduction of bacterial contamination of leafy greens is needed to focus on prevention of contamination events (Gil et al., 2015).

5.1.1 Environmental factors

These factors are related mainly to the specific conditions (growing field and adjacent land characteristics) and climate of the primary production area, which might have an impact in the microbial safety of leafy greens (CAC, 2003; Gil et al., 2015). The conditions at the growing field play a vital role in the microbial safety and each farm represents an exclusive combination of factors that can influence the occurrence and persistence of pathogens in cultivation fields (Strawn et al., 2013a). Additionally,

significant uncertainty surrounds the specific temporal aspects of post-contamination survival sequences and outcomes during leafy greens production despite recent efforts to describe host-pathogen interactions under growth chamber (Patel et al. 2009), greenhouse (Pu et al., 2009) and field conditions (Erickson et al. 2010a; Wood et al. 2010; Moyne et al., 2011).

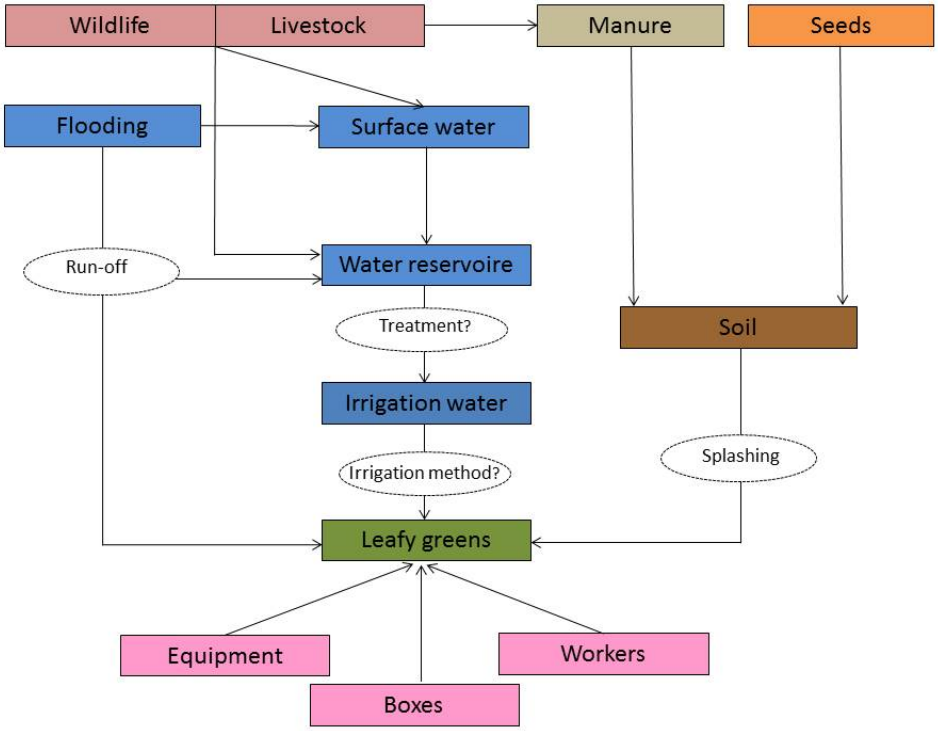

Figure 1.3. Scheme illustrating the main risk factors affecting the microbial safety of leafy greens at primary production level.

- **Field and adjacent land characteristics**

Guidelines and recommendation have established that production of leafy greens should not be carried out in zones where presumptive presence of pathogens would lead to an unacceptable likelihood of transfer to crops intended for human consumption without a validated process kill step (CAC/RCP 1-1969, 2003; CAC/RCP 53, 2003). However, this preventive measure it is not always easy to be achieved because growers are not aware of the activities in the adjacent land or the levels of pathogenic

microorganisms in the soil and the time necessary to reduce them to tolerable levels are not known (Suslow et al., 2003; James, 2006).

With increasing populations and growing necessities for new cultivation fields, fresh produce is often grown in close proximity to urban areas or land used for other types of agriculture, such as livestock production. If crops are cultivated nearby an animal-rearing operation, the product can become contaminated, directly, or indirectly, by animals, run-off, bio-aerosols and vectors associated with the animal operation such as birds, rodents and flies (Brandl, 2006; Gelting et al., 2011).

Cultivation fields located near to urban environment composting operations, areas or concentrated wildlife population areas imply similar hazards for growing crops (Keraita et al., 2003). Topographical characteristics of the fields and contiguous land should also be taken into account in a hazard analysis. Preventive measures to avoid contamination coming from growing field and adjacent land include the development of risk assessment to identify potential point and nonpoint sources (FAO/WHO, 2008). If the growing field is located in a potential hazardous location, intervention strategies focused on the construction of ditches and establishment of buffer areas will help to minimize microbial hazards (Abu-Ashour & Lee, 2000). It is also important to select an adequate crop and crop management practices, including site management that fit a compatible rotation (Leifert et al., 2008).

- **Weather factors**

Leafy greens are usually grown in open field systems and under a wide range of climatic conditions and growing seasons (EFSA, 2014a). The influence of weather factors on the food safety on leafy greens is still not well established and recently several studies have reported the influence of seasonal events and weather conditions on the microbial counts and pathogens on leafy greens and cultivation environment

(Strawn et al., 2013a; Williams et al., 2013; Marine et al., 2015; Park et al., 2015; Ward et al., 2015).

In the same way, climate change is expected to have a significant impact on food safety of leafy greens (Tirado et al., 2010; Liu et al., 2013). This impact has to do with variation in the seasons, modification in the suitability of cultivation areas, changes in crop yields and soil quality (Miraglia et al., 2009). Moreover, climate change has been linked to changes not only in the temperature and the distribution of precipitation (Meehl et al., 2007) but also in the disaster risk patterns mainly by the increase in frequency and intensity of extreme events (Solomon et al., 2007) and alternating periods of floods and droughts (Tirado el at., 2010). These changes are expected to have an important impact on the contamination sources and pathways of pathogens onto leafy greens during the pre-harvest phase (Liu et al., 2013).

It has been reported that an increase in frequency and severity of extreme precipitation events may lead to contamination of soil, agricultural lands, ground, water sources and produce with pathogens originating from sewage, agriculture, urban or industrial settings, among others (Rose et al., 2001; IPCC, 2007). Aon the other hand, after drought periods, soil is compacted and this may result in more severe run-off from manure at livestock farm or from grazing pastures. Run-off has been reported to cause an increased risk of contamination after heavy rainfalls by releasing of large microorganism and a variety of pathogen microorganism to agricultural lands or water courses (Abu-Ashour & Lee, 2000; Orozco et al., 2008; Rajwar et al., 2015). Moreover, when water hits the soil, it can splash microbes already present in the soil up on to the plant (Girardin et al., 2007; Cevallos-Cevallos et al., 2012). Faecal contamination of growing areas has been shown to increase after flooding (Casteel et al., 2006). Variation in rainfall patterns has been shown to have a dramatic effect on decline pattern of

enteric bacterial pathogens in manure and manure-amended soil due to the dehydration-rehydration phenomenon (Ongeng et al., 2011a). A positive correlation between *Salmonella* prevalence and rainfall (mm/day) was reported during a study in southern Morocco (Setti et al., 2009) and a similar study performed in New York state described higher prevalence of *Salmonella* in surface waters when measurable precipitation occurred within 3 days prior to sampling. However, this was observed only in areas of poorly drained soils (Strawn et al., 2013a). A study in Puerto Rico attempted to correlate rainfall from 24 h, 48 h, and 1 week prior to water sampling for faecal coliforms and found no correlation between faecal coliforms and precipitation in any of the 10 sampling sites (Santiago-Rodriguez et al., 2012). The controversial results suggest that rainfall does have some effect on pathogens prevalence in surface waters, not as a direct correlation, but perhaps as a result of the interaction between several factors (i.e. temperature, adjacent land) (McEgan et al., 2013).

An important extreme weather related event that deserves special attention is flooding. Recently, it has been reported that among all outbreaks associated with extreme water-related weather events originated by climate change, heavy rainfall and flooding are by far the most common ones (Cann et al., 2013). These events may have multiple food safety consequences, particularly if the agricultural land is adjacent to livestock farms and industrial and residential areas (Miraglia et al., 2009). It has been reported that flooding and extreme rainfall may lead to pathogen contamination of leafy greens environment (soil, irrigation water and produce) (Casteel et al., 2006; Confalonieri et al., 2007). Additionally, these events may affect persistence, patterns of occurrence and ecology of microorganism not only of pathogens but also leaf or soil microbiota (Tirado et al., 2010). Orozco et al. (2008) reported the presence of *E. coli* and *Salmonella* Newport in tomato samples taken during and after a flooding event.

Another study performed after a flooding event in Kentucky (US) reported that surface water samples collected during flooding had higher levels of *E. coli,* enterococci, *Salmonella*, *Campylobacter*, E. *coli* O157:H7 and adenovirus apart from chemical contaminants in samples taken after 3 months (Ward et al., 2015). Subsequently, fresh produce grown in contaminated land after flooding has been recognized as a potential vehicle for transmission of pathogenic microorganisms (CAC, 2003; EFSA, 2014a), especially in the case of leafy greens that are consumed raw without undergoing any inactivation treatment.

It is known that temperature variations may also affect food safety on the basis of changing the survival or multiplication of some foodborne pathogens (FAO/WHO, 2008). This weather factor has received much attention probably because of the profound effect on the growth and decay rates of bacteria (Ongeng et al., 2015). Several studies have shown a positive relationship between temperature and rainfall and the number of Salmonellosis cases (Zhang et al., 2010). However, it has also been demonstrated that increased UV radiation from sunlight may result in a decrease in potential human risk in water (Zhang et al., 2015), manure (Meays et al., 2015) and leafy greens (Zaafrane et al, 2004). A recent study described a positive correlation between solar radiation and *Salmonella* levels (McEgan et al., 2013). This surprising finding was partially explained by the higher resistant of *Salmonella* to solar radiation than other bacteria (Berney et al., 2006). Relative humidity has also an effect on survival of bacteria in the plant (Dreux et al., 2007). Nevertheless, the mechanism underplaying the relationship between seasonality and foodborne diseases are not fully understood because there is a complex interaction of multiple factors (Liu et al., 2013; Ward et al., 2015). Gorski et al. (2011) reported a noticeable seasonal trend in the prevalence of *Salmonella* in surface water samples from the Salinas Valley of

California. In general, it can be stated that warm temperatures (Holvoet et al., 2014a), greater rainfall (Haley et al., 2009) and high humidity facilitate growth or survival of pathogens on produce (Park et al., 2012). Moreover, the time frame in which temperature or precipitation may affect the microbial load is still not well understood but both short- and long-term weather factors potentially contribute to this relationship (Ward et al., 2015).

5.1.2 Seeds

Seeds could be one potential source of pathogenic bacteria if they are contaminated with foodborne pathogens. They could in theory contaminate the plant and the surrounding soil (Warrimer et al., 2003; Jablasone et al., 2005). A study performed by Van der Linden et al. (2013) demonstrated the survival of *S. enterica* after two years on butter-head lettuce seeds and their subsequent survival and growth on the seedlings.

However, this source of contamination become more important in case of sprout seeds because during sprouting, the environment for bacteria is warm and moisturized which may lead to a rapid bacterial growth. Sprouted seeds are young seedlings obtained from the germination of seeds. They are RTE foods implicated in large outbreaks (EFSA, 2011b). For instance, the large outbreak in 2011 related to *E. coli* O104:H4 that occurred in Germany resulted in more than 4000 illnesses, over 850 cases of haemolytic uremic syndrome and 54 deaths (Frank et al., 2011). The outbreak was linked to the consumption of fenugreek sprouts. The epidemiological investigation suggested that the seeds were contaminated with a pathogen that grew during sprout production (Jung et al., 2014).

5.1.3 Manure and manure-amended soil

When manure is not adequately composted or aged before application, it may introduce faecal pathogenic bacteria, viruses and parasites to leafy greens (Jung et al., 2014). It can also become a contamination source of surface water, ground water and drinking water supplies (Venglovsky et al., 2009). Untreated or improperly treated manure may harbour pathogenic bacteria such as *Salmonella* spp., *E. coli* O157:H7, *C. jejuni*, *Yersinia enterocolitica* and *Clostridium perfringens* (Johannessen et al., 2005; Venglovsky et al., 2009). A pilot study conducted to assess the transfer of *E. coli* from animal slurry fertilizer to lettuce reported concentration of *E. coli* exceeding 2 log CFU/g in 19.0% of the lettuce samples (Jensen et al., 2014). Manure is also of great concern in case of crops that grow close to the soil, like lettuce, because they have a higher probability to get contaminated than crops that grow a few centimetres above the soil (Doyle & Erickson, 2008). Greater intensity of rain events may also lead to vegetable contamination with pathogens by splashing manure particles onto edible portions of the crop or by broadcasting pathogens throughout the field during flooding events (Cevallos-Cevallos et al., 2012). In relation to precipitation, a modelling study showed that the probability of lettuce contamination with *E. coli* O157:H7 from manure-amended soil was significantly correlated with the number of times it rained (Franz et al., 2008a). Moreover, manure piles stored next to growing operations may represent a risk of contamination via run-off, vertebrate and insect vectors, dust or aerosols (Suslow et al., 2003; Brandl, 2006; James, 2006).

Survival of pathogens in manure and manure-amended soil has been demonstrated in several studies (Islam et al., 2004a, b; Franz et al., 2007, 2008a; Semenov et al., 2007; Oliveira et al., 2012). There are a number of factors affecting pathogen survival and prevalence in manure and manure-amended which are related to

the risk of pathogen transfer to the produce: manure type, management during stockpiling, method of application, application rate, frequency of application, and time period between application and planting or harvesting (Hutchison et al., 2004; Jacobsen & Bech, 2011; Ongeng et al., 2014). The prevalence of a range of foodborne pathogens in animal wastes (slurries and manure) from livestock has been reported (Hutchison et al., 2004; Franz et al., 2007). Additionally, environmental conditions play a critical role in the survival of enteric pathogens in soil. A study by Strawn et al. (2013a) described external factors that affected the frequency of isolation and prevalence of enteric pathogens in fruit and vegetable farms. Among them, soil topography, moisture, and proximity to water sources increased the chances of isolating enteric bacteria from vegetable farms. This was consistent with the findings of previous studies, which demonstrated that *Salmonella* and *E. coli* O157:H7 persisted longer in soil with a high moisture level than in dry soil (Ohtomo et al., 2004; Holley et al., 2006).

Several outbreaks have been trace-back to contaminated manure. For instance the large outbreak of *E. coli* O157:H7 associated with spinach in the US in which manure was found to be the source of contamination because molecular typing of isolates from faeces of feral swine matched the outbreak strain (Jay et al., 2007).

5.1.4 Water use during production and irrigation systems

Foodborne illnesses may originate from poor water quality used in fresh produce production. An important factor when considering the quality of water used in fresh produce primary production is the availability of water resources, which is under increasing pressure mainly due to growing population (Painter et al., 2013). Increases worldwide population in the last decades has created increased demands and climate variability causing unpredictability in precipitations (Shah et al., 2014).

Agricultural water has been defined as a major risk factor in the contamination of leafy greens crops eaten raw as salads (EFSA, 2014a). Pathogenic microorganisms associated with irrigation water include bacteria, viruses and parasites (protozoa and helminths) (Allende & Monaghan, 2015). In the US, irrigated produce accounted for nearly half of all foodborne illnesses from 1998 to 2008 (Painter et al., 2013). Farmers use water coming from different sources for field operations and irrigation. There is a need to know the relation between risk factors associated with the transfer coefficients for pathogens by source, concentration and use (Uyttendaele et al., 2015). Sources of irrigation water can be generally ranked by the microbial contamination hazard that in order of increasing risk are: potable or rain water, ground water from deep wells, groundwater from shallow wells, surface water, and finally raw or inadequately treated wastewater (Leifert et al., 2008).

The contamination of leafy greens with enteric pathogens from irrigation water is associated with either the source/type of water or the irrigation method (Steele & Odumeru 2004; Leifert el al., 2008). Pathogenic bacteria such as pathogenic *E. coli* and *Salmonella* spp. have been reported from surface water sources (Forslund et al., 2012; Holvoet et al., 2014a; Delbeke et al., 2015a). These pathogens could be introduced into the crop growing environment through irrigation (Strawn et al., 2013a,b). Mitra et al., (2009) reported that *E. coli* O157:H7 was internalized in spinach plant irrigated with contaminated water. One on the leaf or inside, these bacteria have been shown to be capable of surviving and persisting in the plant and the soil for long periods (Ibekwe et al., 2007; Barker-Reid et al., 2009; Mitra et al., 2009; Poza-Carrion et al., 2013). There is even evidence that when these bacteria, whose main host are mammals, are exposed to surface water before introduction to a plant surface, they are better able to survive and persist on plant surfaces. This is probably due to a stress response that causes the

bacteria to produce structures that mediate bacterial attachment and exopolysaccharides which protect them from desiccation and UV exposure (White et al., 2006; Lapidot & Yaron, 2009).

Regarding water sources, water coming from different sources and qualities of water is used for irrigation, with each of these having a different propensity to result in microbiological contamination of the crops. In addition, the method of irrigation plays an important role in the mode of contamination and transfer of bacteria to the crop (Uyttendaele et al., 2015).

A surface area of 70 % of Spain is subject to a semi-arid climate with recurring droughts and strong seasonal variability, especially the South East is facing increasing water shortages (Fuentes, 2010). Most river basins identified as being particularly affected by water scarcity are characterized by intensive irrigation use (Terceño-Gómez et al., 2009). The natural water resources in Spain were estimated around 100.000 hm^3/year, 74% corresponding to surface water and 26% underground water, while the present demand is estimated as 35.310 hm^3/year (Pedrero et al., 2010).

Although a big part of the water used for irrigation of the leafy greens comes from water reservoirs where rainwater and surface water is stored for further used in the field, other important part comes from treated wastewater which is treated to be reused (Keraita et al., 2010). As an example, almost 60% of the reclaimed water produce was reused (Pedrero et al., 2010). Rainwater is generally of relatively good microbial quality and it depends in part on the means by which it is collected or transported. However, during storage the water can be contaminated with pathogenic bacteria and protozoan parasites because of the presence of bird, insect, and animal droppings (Ahmed et al., 2002). Surface water includes lakes, rivers, creeks, ponds, and springs that come to the surface. Very often surface waters are contaminated due to discharges of (treated)

wastewater, storm water runoff, livestock or wildlife faeces (Uyttendaele et al., 2015). It is not surprising that the study performed by Mañas et al. (2009) found that lettuce irrigated with drinking water had lover *Salmonella* and coliform levels that did lettuce irrigated with treated wastewater. In Spain, reclaimed water is usually of very poor physicochemical and microbiological quality and, consequently, requires further treatment prior to use in irrigation, (Pedrero et al., 2010). Hence, its use is not recommended especially in the case leafy greens irrigation because the water contacts directly with the edible tissue.

In relation to irrigation methods used for leafy greens production, they vary depending on region and crop and the potential microbial risk entailed by water vary also with the irrigation method (Stine et al., 2005). In Spain, modernization of irrigation techniques have occurred during the last decades; flood irrigation – the least efficient technique has declined and it has been progressively replaced by drip and sprinkler irrigation covered 68% of the surface and accounted for about 58% of water supplied (Fuentes, 2010). The expansion of drip irrigation and sprinkler occurred especially in the southern half of Spain, where replacing of ineffective systems with more efficient irrigation systems has been carried out (MARM, 2010). Sprinkler irrigation is widely used for leafy greens production (**Picture 1.3**). This type of irrigation water is applied in the form of a spray. It can facilitate the contamination of ground crops because the edible portion of the produce is directly exposed to water and it may be a particular problem especially when applied close to the harvest time (Wood et al., 2010). Additionally, splashing caused by the sprayers can provoke recontamination of the crop surface from the soil (Girardin et al., 2005; Cevallos-Cevallos et al., 2012). In contrast, drip irrigation represents lower risk because the water may not be transferred to the

fresh produce as the contact between water and produce is restricted (López-Gálvez et al., 2014; Uyttendaele et al., 2015).

Picture 1.3. Sprinkler irrigation during production of baby spinach.

In relation to water quality, little is known regarding the microbial status of water used for leafy greens production (Allende & Monaghan, 2015). The available literature indicates that generic *E. coli* levels and prevalence of pathogenic foodborne bacteria in irrigation water vary depending on a number of factors including seasonality, geographical location and weather conditions, among others (Gil et al., 2015; Uyttendaele et al., 2015). Several longitudinal studies have been performed in order to determine the impact of irrigation water on the microbial safety of fresh produce, however important variations among studied countries have been reported (Allende & Monaghan, 2015). In the UK, Tyrrel et al. (2006) reported that most of growers did not irrigate with faecal contaminated water as they would meet European Union Drinking Water Standard. In Belgium, irrigation water of eight lettuce farms was monitored and a high prevalence (75%, n = 120) of *E. coli* was found with 65% of the samples having *E. coli* levels ≥1 log CFU/100 mL while 26% of the samples showed *E. coli* counts ≥2 log CFU/100 mL, which is above most of the irrigation water-quality standards. Additionally, 35% of the collected samples were positive for at least one pathogen

(*Salmonella*, *Campylobacter* or Shiga toxin-producing *E. coli* (STEC)) (Holvoet et al., 2014a).

Another water-related source of contamination may be the use of contaminated water to prepare pesticide solutions since bacterial pathogens such as *Salmonella* or *E. coli* O157:H7 have been reported to survive or even multiply in some pesticide formulations and to be transferred from pesticide solution to fresh produce (Guan et al., 2001; Stine et al., 2011; Dobhal et al., 2014).

Therefore, water used during production plays an important role in the contamination of leafy greens and a number of fresh produce related outbreaks have been associated with poor quality water used during fresh produce cultivation (Uyttendaele et al., 2015). Faecal contaminated irrigation water has been implicated as either a possible source, or a likely source of pathogen contamination of fresh, raw consumed fruits and vegetables (De Keuckelaere et al., 2015). For instance, irrigation water has been responsible for several outbreaks related to fresh produce (Greene et al., 2008; Behravesh et al., 2011). Regarding leafy greens (**Table 1.2**), irrigation water was related to the 2006 multistate *E. coli* O157 outbreak associated with spinach (Gelting et al., 2011) and iceberg lettuce contaminated with *E. coli* O157 caused a large outbreak in Sweden in 2005, possibly due to surface water used for irrigation (Söoderström et al., 2008). Another outbreak occurred in June 2013 in Sweden caused by fresh salads contaminated with Enterohaemorrhagic *E. coli* (EHEC) probably by irrigation water with, although this could not be established (Edelstein et al., 2014). In the same year, a verocytotoxin-producing *E. coli* outbreak associated with watercress was attributed to either wildlife or contaminated runoff water (Public Health England, 2014). Several microbiological surveys to assess the prevalence of pathogens on farm environments have recovered *Salmonella* from irrigation water (Gorski et al., 2011; Micallef et al.,

2012). It has been reported that surface water used for irrigation consistently appear to be the mayor reservoir for *Salmonella* spp. (Micallef et al., 2012; Strawn et al., 2013b). Shiga-toxin producing *E. coli* (STEC) was also isolated from surface water source used for irrigation (Strawn et al., 2013a) and in watersheds near mayor vegetable production areas (Cooley et al., 2007).

However, despite of all the available research and spectrum of results, the establishment of appropriate guidelines for pre-harvest application of irrigation water is still difficult (Matthews, 2014).

5.1.5 Animal contamination

The contamination of leafy greens with pathogenic microorganism can occur directly or indirectly via animals (Gil et al., 2015). The risks posed by livestock and wild animals depend not only on prevalence and amount of pathogens carriage in the animal hosts but also on the degree of interaction between animals and cultivation environment (Alam & Zurek, 2006; Khaitsa et al., 2006; Jay et al., 2007; Oporto et al., 2008). Domestic animals can be separated from growing fields but to control the access of wild animals (e.g. frogs, lizards, snakes, rodents, birds, etc.) can be very difficult (Harris et al., 2003). The contamination event can occur if the pathogens enter the cultivation environment through direct deposition of faeces to the soil if they pass by growing areas (EFSA, 2014a). Animals can shed foodborne pathogens in the absence of signs of illness acting as vectors of *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes* and *Campylobacter* (Moncrief & Bloom, 2005). This section is focused on pathogenic *E. coli* strains and *Salmonella* spp.

Cattle are known to be the main reservoir host for several pathogenic *E. coli* strains (Berry & Wells, 2010). Reported prevalence of *E. coli* O157 in beef cattle on rangeland and pasture can vary from 0.9% (82/9122) to 18% (9/50) (Benjamin et al.,

2015). The isolation of *E. coli* O157:H7 and other STEC like O26, O103, O111, and O145 from cattle have been repeatedly reported (Jenkins et al., 2003; Pearce et al., 2006; Murphy et al., 2007; Fremaux et al., 2008; Karmali et al., 2010). A recent study examined the impact of proximity to a beef cattle feedlot on *E. coli* O157:H7 contamination of leafy greens. At all plot distances, the pathogen was recovered from the plants highlighting that current leafy greens field distance guidelines of 120 meters may not be adequate to limit the transmission of *E. coli* O157:H7 to produce crops planted near concentrated animal feeding operations (Berry et al., 2015). Furthermore, *E. coli* 0157:H7 has also been isolated in non-bovine species (deer, sheep, goat) and a number of domestic and wild animals including horses, pigs, chickens, turkeys, dogs and rats (Chapman et al., 1997; Nielsen et al., 2004).

On the other hand, *Salmonella* spp. have also been isolated from various species of animals that can come into contact with growing fields, including wild boar (Vieira-Pinto et al., 2011; Zottola et al., 2013), deer, birds (Benskin et al., 2009; Tsiodras et al., 2008), rabbits (Vieira-Pinto et al., 2011), rats (Lapuz et al., 2008), flies (Pava-Ripoll et al., 2012) and reptiles such as lizards, chameleons, turtles and snakes (Beuchat et al., 2006). Although wildlife has been repeatedly suggested as a cause of contamination of fresh produce at pre-harvest level, the trace-back contamination from animal to leafy greens has been confirmed only a few times (Sagoo et al., 2003; Jay et al., 2007).

Last but not least, insects have been reported as a possible pathway of contamination as they are frequently found in manure piles, feedlots and other habits near fields of leafy greens (Martínez-Vaz et al., 2014). Investigations on the role of filth flies as insect vectors in the contamination of fresh produce revealed that a large number of flies caught near agricultural fields were carriers for *E. coli* O157:H7 and transfer was reported under laboratory conditions (Talley et al., 2009). Wasala et al. (2013)

investigated the deposition of *E. coli* O157:H7 by domestic flies onto spinach and demonstrated the multiplication of bacteria in regurgitation spots found on the plant surface which may have provided a nutrient source for the survival of pathogenic *E. coli* on spinach leaves. Taken together these findings, they suggest that insect vectors, especially houseflies, may be vehicles for the transmission and persistence of foodborne pathogens in leafy green.

5.1.6 Sources of contamination associated with harvesting practices

- **Equipment**

Leafy greens are usually mechanically harvested by means of a harvesting machine with a blade used to cut the leaves from the stem (ANR, 2012). The proximity of the blade to soil during harvest, and the adherence of soil to lower portions of the plant, may result in a great potential for soil contact with the cutting blade. Thus, the cutting blade represents a critical vehicle for pathogen transfer (Yang et al., 2012). In fact, several studies have demonstrated that a single coring knife artificially inoculated with *E. coli* O157:H7 could contaminate lettuce heads during harvesting (Taormina et al., 2009).

Additionally, the harvesting machine could pick up faecal deposits in the field contaminating large volumes of harvested produce (Jay et al., 2007). Furthermore, containers, boxes and conveyor belts could represent a source of contamination as suggested from previous research studies (Prazak et al., 2002; Johnston et al., 2006). Ailes et al. (2008) found that when compared to produce samples taken directly from the field, those items collected from the packing bins had over a six-fold increase in likelihood of *E. coli* contamination and only a four-fold increase in likelihood for samples that originated from the box or conveyor belt. Considering these previous

results, the hygiene and equipment sanitation during harvest is a critical point for microbial safety of leafy greens (EFSA, 2014a).

- **Farm workers**

The lack of good hygienic practice by the farm workers can lead to cross-contamination during the harvest stage (Gil et al., 2015). For example, hygiene and provision of instructions on the proper use of gloves or hand washing facilities is necessary to prevent the transfer of pathogens to leafy greens (Suslow et al., 2003; James, 2006). Cross-contamination via food handlers is a main factor since it has been recently reported that murine NoV spiked onto iceberg lettuce could be transferred to the fingertips of nitrile gloves after touching the produce for 5 seconds (Verhaelen et al., 2013).

Poor personal hygiene was identified as a contributing factor in outbreaks of gastroenteritis where NoV was assigned as the causative agent (Noda et al., 2008). Moreover, it has been demonstrated that NoV can be shed by individuals which are asymptomatically infected without being aware (Atmar et al., 2008; Phillips et al., 2010) or that can also be shed for several days after the symptoms have resolved (Atmar et al., 2008; Zelner et al., 2013). This capacity for NoV to be shed in the absence of symptoms is a significant factor underlying the hazard of these highly contagious viruses, and clearly indicates the absolute necessity for hand hygiene at all times by all food handlers (EFSA, 2014a).

Additionally, leakage from portable toilets to fields and in-field defecation has also been identified as potential source of contamination (Suslow et al., 2003). Last but not least, training is crucial to any food safety systems since poor staff training in food hygiene is a real threat to the safety of food; hence effective training is an important

prerequisite to successful implementation of a food safety management system (Arvanitoyannis & Kassaveti, 2009).

5.2 Sources and factors affecting post-harvest contamination of leafy greens

Leafy greens processing operations involve the application of several unit operations, which can provide opportunities for cross-contamination whereby a small lot of contaminated product may be responsible for contamination of a large lot (IFPA, 2001; FDA/CFSAN, 2008). It is recommended that processors ensure that their suppliers (growers, harvesters, packers, and distributors) adopt the principles outlined in the Code of Hygienic Practice for Fresh Fruits and Vegetables (CAC/RCP 1-1969, 2003; CAC/RCP 53, 2003; Suslow, 2003). Preventive sanitation programs such as GMPs, and Sanitation Standard Operating Procedures (SSOPs), if properly implemented are likely to minimize the chance of contamination by pathogenic bacteria, viruses, and parasites (WGA, 2012).

5.2.1 Environmental factors

In this section we refer to the specific conditions of transportation, processing, storage and retail areas, which might have an impact on the safety of the leafy greens (CAC, 2003). Temperature is one of the key factors in leafy greens processing plants (EFSA, 2014a). Many research papers described the relevance of low temperature as a strategy to avoid/reduce bacterial growth of foodborne pathogens in leafy greens (Oliveira et al., 2010; Sant'Ana et al., 2012; Posada-Izquierdo et al., 2013). Cold storage drastically reduces the growth rates of most human pathogens (FDA, 2009b). Moreover, temperature is one of the most important environmental parameters affecting both food quality and safety of fresh produce although it must be maintained at levels suitable for

the each specific produce being cooled (Coetzer, 2006). In general, fresh produce that is not temperature sensitive should be preserved at temperatures below approximately 5ºC to reduce the proliferation of spoilage organisms and human pathogens. However, maintaining a consistently low temperature throughout the distribution chain of leafy greens is challenging (Zeng et al., 2014). The opening of truck doors during loading and unloading, outside temperature extremes, retail storage and display conditions can contribute to temperature fluctuations. Several studies conducted in Canada, Japan, and Belgium have assessed the growth of *E. coli* O157:H7 and *L. monocytogenes* under real-time temperatures during pre-harvest, transportation and retail sale of RTE salad greens (Koseki & Isobe, 2005; Rediers et al., 2009; McKellar et al., 2012). These studies together with the recent research of Zeng et al., (2014) reported the impact of fluctuating temperatures on microbial growth for RTE leafy greens. They highlighted temperature abuse as the main contributing factor to foodborne outbreaks (Tirado & Schmidt, 2001; Zeng et al., 2014). They emphatized that the disruption of the cold chain can cause a substantial increase in microbial load (Rediers et al., 2009). Hence, maintenance of the cold chain is of particular importance for fresh produce because of the absence of thermal treatment before consumption (Franz et al., 2010; Gil et al., 2015).

In general, pathogens such as *Salmonella* can be controlled in leafy greens by ensuring that these products are stored at a temperature below 7 ºC (EFSA, 2014a). Oliveira et al. (2010) observed that the population of *Salmonella* decreased in shredded romaine lettuce approximately 1 log unit after 10 days at 5 ºC, while it increased about 2 log units after 3 days at 25 ºC. Another study carried out on lettuce reported that *E. coli* O157 population increased by more than 2 log at 30 ºC within 8 hours (McEvoy et al., 2009). Additionally, internalization of *Salmonella* and pathogenic *E. coli* in the

detached leaves can also occur due to the impact of post-harvest operations (Gómez-López et al., 2013). Furthermore, survival of bacterial pathogens such as *Salmonella* and STEC can occur on leafy greens under certain conditions of storage (Tomás-Callejas et al., 2011; Delbeke et al., 2015b).

5.2.2 Equipment

Processing of fresh produce comprises a succession of different operations where contamination and cross-contamination can occur (Pérez- Rodríguez et al., 2011; Gil et al., 2015). Although, some studies have reported that conveyor belts, centrifugation and filling operations are not usually significant sources of contamination (Garg et al., 1990), others studies have found that numbers of natural microbiota (total count) increased about 1 log unit CFU/g after centrifugation (Allende et al., 2004). Surfaces of processing equipment have been recognized as sources of microbial contamination and recontamination (Lehto et al., 2011). In terms of equipment surfaces, a study aimed at assessing *E. coli* O157:H7 cross-contamination during leafy greens processing found that the conveyor belt and the shredder were microbial 'hot spots', as exudate from shredded lettuce was visible on the cutting wheel and discharge chute of the shredder, as well as on all product contact areas of the conveyor (Buchholz et al., 2012a). This likely enhanced *E. coli* O157:H7 transfer from lettuce to the stainless steel and polyurethane surfaces of the shredder and conveyor belt (Moore et al., 2003).

Other publications have reported that shredding induces mechanical injury in the tissue and causes physiological disorders due to the disruption of the plant tissues, breaking protective epidermal layers and releasing nutrient-rich vascular and cellular fluids (Artés & Allende, 2005; Martinez et al., 2008). Besides disruption of the physiological state, cut surfaces produces large amounts of nutrients that can be used by

the microorganisms inducing localized microbiological proliferation (Doyle & Erickson, 2008) and internalization through wounds (Erickson, 2012).

Additionally, the machinery used for cutting needs to be cleaned and disinfected at regular time intervals to avoid organic residues accumulation (Artés & Allende, 2005). Another important issue is biofilm formation, which is difficult to remove even with the cleaning practices routinely used in the food industry and it may remain and survive in the plant environment (Romanova et al., 2007). Poorly cleaned and maintained equipment can harbour microorganisms, including pathogens, and provide a reservoir of contamination (Stafford et al., 2002). *E. coli*, *L. monocytogenes* and *Salmonella* spp. have been isolated from conveyor belts and cooler surfaces (Duffy et al., 2005; Johnston et al., 2006). Contaminated shredding equipment was identified as the source of contamination in an outbreak of salmonellosis attributed to shredded lettuce produced in a commercial setting (Stafford et al., 2002). When *E. coli* O157:H7 was experimentally inoculated on leafy greens, 90% of the inoculum was shed to the disinfectant-free water, contaminating the surfaces of shredders, conveyor, flume tank, shaker table and dewatering centrifuge and highlighting equipment as a potential source of cross-contamination (Buchholz et al., 2012a). Furthermore, leafy green processing typically involve the use of various types of conveyor belt systems manufactured from different belting materials (i.e. high density polyethylene, polypropylene and acetyl). Generally, all conveyor belts are prone to microbial build-up and the subsequent transfer of microorganisms to incoming product over time. The newer smooth continuous belts, which can be more easily cleaned and sanitized, are now generally preferred over the older interlocking belts that must be dissembled and then manually cleaned and sanitized (Sinha et al., 2010).

In addition, the likelihood for virus contamination of produce items to spread via cross-contamination through contact with food processing or preparation surfaces also exists although unlike bacterial contaminants multiplication of viruses outside the host cannot occur (Escudero et al., 2012).

5.2.3 Washing

Washing is a key intervention step in the processing of leafy green and it is aimed to remove dirt, foreign materials, tissue fluids from cut surfaces, and microorganisms. As RTE leafy greens do not undergo intensive inactivation or preservation treatments during processing, washing is the only processing step to reduce the microbial load on leafy greens (IFPA, 2001). However, water use during processing of leafy greens has been identified as a potentially important source for cross-contamination with faecal indicator organisms (e.g. *E. coli*) and human enteric pathogens (Allende et al., 2008; Luo et al., 2011; Buchholz et al., 2012a; Holvoet et al., 2012; Rodriguez-Lázaro et al., 2012; Shen et al., 2013). Thus, the quality of the wash water is very important as washing with water of unsatisfactory quality can lead to cross-contamination (Allende et al., 2008).

Leafy green contamination and cross-contamination during washing were established in a recently simulation study carried out by Holvoet et al. (2014b) who reported that during washing, a small proportion (<1.5%) of the microorganisms (whether *E. coli*, *E. coli* O157, MS2 phage or murine Norovirus) were transferred from the water phase to lettuce, highlighting the vulnerability of leafy greens to cross-contamination by enteric bacteria and viruses during the washing stage. However, this experiment was performed without sanitizers. Some studies have highlighted the importance of maintaining water quality during washing using appropriate sanitizers to help minimizing the potential microbial contamination of processing water and

subsequent the cross-contamination of the product (FDA, 2008; Zhang et al., 2009; López-Gálvez et al., 2010a). Thus, sanitizing agents are recommended to maintain the microbial quality of the water and prevent cross-contamination of the product, in spite of their limited direct antimicrobial effect on microbes attached to the produce (Gil et al., 2009) and on forming biofilms on leaf surfaces (Niemira & Cooke, 2010). The efficiency of several sanitizers against different enteric pathogens has been proven. For instance, sodium hypochlorite (NaOCl) and peroxyacetic acid (PAA) were shown to reduce NoV in leafy greens (Baert et al., 2009) and in process water (Baert et al., 2008). However, other results showed that NaClO and Chlorine dioxide $(ClO)_2$, which has been postulated as an alternative, were not able to control *E. coli* cross-contamination after washing because of the location of the bacterial cells in clusters or tissue stomata protected from sanitizers (López-Gálvez et al., 2010a). Additionally, the effect of sanitizers can be reduced due to the increasing presence of organic matter in the wash water during a production cycle which demonstrates the premise for a disinfectant residual during washing by means of water monitoring and dosing of the disinfectant (Gil et al., 2009; Banach et al., 2015).

On the other hand, the reaction of chemical disinfectants with water matrix constitutes lead to the formation of derived by-products (DBPs). In particular, the challenges surrounding the presence of high amounts of organic matter and the resulting DBPs have raised scientific, industrial, and political concerns (Gil et al., 2009; Ölmez et al., 2009). In case of chlorine, problems arise as a result of the potential health and environmental concerns due to the formation of carcinogenic, halogenated DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs) during its application (Gil et al., 2009; Ölmez et al., 2009; López-Gálvez et al., 2010b).

Due to the problems associated with wash water disinfection (i.e. cross-contamination, formation of potentially harmful chlorinated by-products, effect of organic matter accumulation), extensive research is being carried out aimed at investigating the effectiveness of alternative disinfection treatments to control the washing process and prevent cross-contamination. In this line, among the proposed alternative disinfection treatments, the utilization of electrochemical water disinfection could help to reduce the chlorine requirements in the washing tank while helping to maintain a reasonable safety level of fresh-cut products by avoiding cross contamination (López-Gálvez et al., 2010a). A study carried by López-Gálvez et al. (2012) aimed to assess the suitability of electrochemical treatment using boron-doped diamond electrodes showed its potential for water disinfection and organic matter reduction even without adding NaCl.

In conclusion, several parameters can affect the efficacy of water disinfection treatments. Sanitizers should be used to maintain wash water quality but, in addition to its microbiological and chemical safety, the effect on product quality is essential to consider in parallel with legal aspects when selecting disinfectant and method. Thus, additional research into the influencing factors is critical for the appropriate selection of disinfectants and application method.

5.2.4 Dewatering step

After fluming and washing, excess of water must be removed before packing through the use of shaker tables, blowers, rotating conveyors, or centrifugal dryers to maintain product quality and an acceptable shelf life (Gorny et al., 2002). It has been reported that leafy greens at the bottom of the bag if there is water condensation, showed higher total counts than those on top, emphasizing the importance of water

removal (Valentin-Bon et al., 2008). All drying methods are product dependent with the end goal of product quality and shelf life preservation (Sinha et al., 2010).

One of the most used methods to dewater leafy greens is centrifugation by spinning to force water to the outside of a collection vessel. The time and speed of centrifugation are key parameters to adjust each product. Thus, centrifugation strongly affects product quality and shelf life as the inadequate drying may result in fast quality deterioration and microbial growth and the excessive centrifugation may cause cellular damage (Ahvenainen, 2000). Additionaly, it can cause products to leak fluids after packaging and this greatly reduces quality (Luo & Tao, 2002). Regarding the potential risk that centrifugation represents for microbial safety of leafy greens, Tomás-Callejas et al. (2012) demonstrated that the centrifugal force applied during this step removed a proportion of attached cells from the leaf surface. Consequently, this step represents a potential risk for cross-contamination transference to product and equipment prior to packaging. Additionally, these results further suggest that the centrifugation effluent water could be used as a sampling point to evaluate lot contamination by low levels of pathogens and cross-contamination during leafy greens processing (Tomás-Callejas et al., 2012).

Many leafy greens are too delicate to withstand centrifugal drying, therefore forced air in a semi-fluidized bed can be used to strip water away from products. It is most effective on product pieces that have smooth surfaces allowing water to be swept away from the product. Highly textured surfaces, with nooks and crannies are much more difficult to dry via this method. Any forced air used in such types of operations must be filtered to avoid the contamination of the products (Sinha et al., 2010). However, its main inconvenience is the low efficiency to dry high volumes of product (Turatti, 2011).

5.2.5 Packaging and storage

The final operation in the processing of leafy greens takes place in the assembly and packaging room, ideally separated from the washing section. Packing is performed around a vertical tube at the top of which is the associated weight- based portion control machine (Gil et al., 2015). Packaging under hygienic controlled conditions immediately after drying has an important role for the microbiological protection of fresh-cut produce (FAO/WHO, 2008; Turatti, 2011). The correct combination of packaging material, produce weight, and gas composition within a package are critical components, which must be determined for each product to maintain product quality and safety and extend product shelf life (Jacxsens et al., 2003).

Packaging of leafy vegetables maintains a high humidity, which is an important factor for survival and growth of pathogens (Brandl & Mandrell, 2002; Dreux et al., 2007). In general, the decline of pathogens has been described for low humidity and growth for saturated humidity (Brandl & Mandrell, 2002).

Modified atmosphere packaging (MAP) technology is largely used for minimally processed fruits and vegetables including RTE leafy greens. This technique is aimed not only to extend the shelf life of leafy greens (Jacxsens et al., 2001; Abadias et al., 2012) but also to inhibit or retard the growth of spoilage and some pathogenic microorganisms, particularly due to the low O_2 concentration (Zang et al., 2015). Leafy vegetables have a high respiration rate, which increases further by tissue damage caused during processing. Packaging in atmosphere with depleted O_2 and/or enriched CO_2 levels can reduce respiration, delay ripening, decrease ethylene production, retard textural softening, slow down compositional changes associated with ripening, thereby resulting in the shelf life extension (Rico et al., 2007). The recommended percentage of O_2 in a modified atmosphere for fruits and vegetables for both safety and quality ranges

between 1 and 5% (Sandhya, 2010) with levels of CO_2 between 3 and 10 % (Jacxsens et al., 1999; Jacxsens et al., 2001). However, pathogens such as *Clostridium perfringens*, *C. botulinum* and *L. monocytogenes* are minimally affected by CO_2 levels below 50%. There is a concern that by inhibiting spoilage microorganisms, a food product may appear edible while containing high numbers of pathogens that may have multiplied due to a lack of indigenous competition (Zagory 1995; Phillips, 1996). Additionally, the practical implications of interactions between modified atmosphere and storage temperatures/times on survival and growth of enteric pathogens are controversial. On one hand, there appear to be no effects on MAP on growth of *E. coli* O157:H7 (Abadias et al., 2012; Posada-Izquierdo et al., 2013) but enhanced effects on *Salmonella* spp. growth at abuse temperatures (i.e. 15 or 25 ºC) (Oliviera et al., 2010; Sant'ana et al., 2012). On the contrary, significant enhanced effects on *L. monocytogenes* growth have been reported at modest abuse temperatures of 8 ºC but no without oxygen depletion (Francis & O'Beirne, 1997, 2001). A recent study aimed at determining the effect of O_2 depleted atmospheres on *L. monocytogenes* growth showed the potential hazardous effect by facilitating its growth under mild abuse temperatures (i.e. two days at 4 ºC, two days at 8 ºC and subsequent storage at 7 ºC) and extended storage life (O'Beirne et al., 2015).

It became clear that MAPs have the benefit of a general reduction in the rates of metabolic processes and the retardation of senescence of leafy greens. However maintenance of low temperature throughout the leafy greens processing and distribution chain and the used of modest shelf life are critical issues to ensure the microbial safety (O'Beirne et al., 2015).

5.2.6 Workers

Lack of compliance of workers with Good Manufacturing Practices (GMP) and Good Hygiene Practices (GHP) is a risk factor for leafy green processing (EFSA, 2014a). It is recommended to have standard enforceable policies and provide training in sanitation to all employees (EC Nº 852/2004). People known or suspected to be suffering from an illness likely to be transmitted through fresh leafy vegetables should not be allowed to enter any food handling area (FAO, 2003). If a worker has a potential source of contamination such as cuts or wounds, these should be covered by suitable waterproof dressings before permitted to continue working (Ritenour et al., 2010; WGA, 2012).

Wachtel et al. (2003) showed that lettuce leaves could be easily contaminated via contaminated hands and Espinoza-Medina et al. (2006) detected *Salmonella* spp. on workers hands. An outbreak of hepatitis A was implicated by an infected food handler shredding lettuce (Harris et al., 2003). Another outbreak in the US was caused by shredded lettuce contaminated with *Shigella* by a food handler (Davis et al., 1998).

6. Current tools to assess microbial safety

6.1 Pathogen monitoring

One way for monitoring the safety of fresh products along the produce chain is the establishment of microbial testing at different points throughout the production process in order to detect foodborne pathogens and verify the acceptance of a lot (Pérez-Rodriguez et al., 2014). Nevertheless, survey studies designed to investigate the presence of enteric pathogens in fresh produce have shown that contamination occurs infrequently and at low levels (Mukherjee et al., 2004, 2006; Bohaychuk et al., 2009;

Koseki et al., 2011; EFSA, 2014a). Contamination is usually heterogeneously distributed so that the probability of pathogen detection dramatically decreases (Pérez-Rodriguez et al., 2014). Random sampling, when available, could be a simple approach to provide detection of pathogen positive samples. However, systematic sampling is, in many cases, more effective in detecting clusters of microorganisms (Jongenburger et al., 2011). Thus, there is a lack of information about the adequate sampling method to follow in leafy greens as well as the evaluation between-lots and within-lot variability to detect bacterial pathogens and enteric viruses. A recent study carried out by Pérez-Rodriguez et al. (2014) aimed to determine the effect that the concentration and prevalence of several pathogens (*L. monocytogenes*, *Salmonella* spp. and enteric pathogenic viruses) in lettuce have comparing two different sampling plans. This study concluded that pathogen testing should not be considered standalone as a reliable tool for microbial safety assurance in the vegetable industry. Other control measures should be applied to minimize the presence of enteric pathogens in vegetable products.

Additionally, detection of pathogens is expensive, time consuming, and complex (Savichtcheva & Okabe, 2006). Consequently, pathogens are most of the time not directly monitored in fresh produce production and processing and instead, indicators microorganisms are routinely monitored by the industry, environmental agencies and public health organizations (EFSA, 2014a). However, the extent of correlation among themselves and predictive value of these hygiene indicators for pathogen's presence has not been thoroughly established or quantified (Holvoet et al., 2014a).

6.2 Indicator microorganism

While produce contamination with enteric foodborne pathogens is of high public health and economic concern, the contamination events are relatively rare, thus

requiring intensive but also expensive sampling and testing efforts that makes the use of faecal indicators, particularly *E. coli*, a more practical approach (Allende & Monaghan, 2015).

According to EFSA (2014a), the term 'indicator microorganism' is suggested for those marker organisms whose presence in given numbers points to 'inadequate processing' for microbial safety. Thus, a positive test for indicator microorganisms does not necessarily mean presence of pathogenic microorganisms in a specific commodity. This contrasts with the term 'index' microorganism that stands for marker microorganisms whose presence in numbers exceeding established limits indicates the possible occurrence of ecologically similar pathogens (Mossel et al., 1995; Smith & Schaffner, 2004; EFSA, 2014a). To eliminate the ambiguity in the term microbial indicator, the following three groups are now recognized (**Table 1.4**): i) general (process) microbial indicators, ii) faecal indicators such as *E. coli*, iii) index organisms and model organisms (Odonkor & Ampofo, 2013). In short, index markers indicate a potential health risk, whereas indicators reveal process failure (Leclerc et al., 2001).

Table 1.4. Definitions for indicator and index microorganisms of public health concern.

Group	Definition
Process indicator	A group of organisms that demonstrates the efficacy of a process such as total heterotrophic bacteria or total Coliforms for chlorine disinfection.
Faecal indicator	A group of organisms that indicates the presence of faecal contamination such as the bacterial group thermotolerant Coliforms or *E. coli*. Hence, they only infer that pathogens may be present.
Index and model organisms	A group/or species indicative of pathogen presence and behaviour respectively such as *E. coli* as an index for *Salmonella* and F-RNA coliphages as models of human enteric viruses.

The fresh produce industry has used indicator monitoring with the aim of determining the microbial quality and hygiene status their products. In particular, in the framework of verification of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) in leafy green production and processing (Wilkes et al., 2009; Ferguson et al., 2012). Ideally, indicators should be present in the intestinal tract of the same animal as the pathogens; they should be present only in contaminated samples and not in uncontaminated ones; they should have similar survival patterns as pathogens outside the host; they should not be able to grow and proliferate in the environment; they should be easily detectable; they must be of low risk to the person conducting the analyses and, ideally, they should be relatively cheap to use (Ishii & Sadowsky, 2008; Ferguson et al., 2012).

There are a wide variety of bacterial genera, groups, and species, viruses and bacteriophages that have been used or proposed for use as indicator microorganism (Bae & Wuertz, 2012). It is common practice to monitor the presence and levels of indicator bacteria such as total bacteriophages, coliforms, enterococci, enterobacteriaceae and *E. coli* (Suslow et al., 2003). These microbial parameters are often used to indicate insufficient sanitary quality, potential faecal pollution or failures in control measures. Nevertheless, contradictory results have been reported about the efficacy of index or indicator microorganism in predicting the presence and/or prevalence of human pathogens such as *Salmonella* in surface water (Chandran et al., 2011; McEgan et al., 2013) or in fresh produce (Ceuppens et al., 2015). In general, it can be assumed that correlations between pathogens and index/indicator are moderate and that high correlations may be temporal, random, site or time specific (Payment & Locas, 2011; McEgan et al., 2013). Therefore, the complex nature of the index or indicator and pathogen relationship makes predicting the levels of pathogens through index/indicator

microorganisms challenging; simple, linear relationships cannot be relied upon for predicting pathogen levels from indicator populations (McEgan et al., 2013).

6.2.1 *E. coli* as indicator

Among the mentioned indicator bacteria, generic *E. coli* is the most used indicator microorganism in the fresh produce industry because of the dual purpose: it can act as an 'indicator' to verify GAP and GMP and absence of significant faecal contamination and as an 'index' microorganism providing evidence of an increased probability of potential contamination by ecologically closely related pathogens (Mossel et al., 1995; Odonkor & Ampofo, 2013).

It is generally assumed that *E. coli* is an effective index organism, but there is little evidence for a definitive correlation between the presence or levels of *E. coli* and the presence of enteric pathogens such as *Salmonella*, pathogenic *E. coli* strains and enteric viruses (Busta et al., 2003).

The available data related to *E. coli* levels and pathogens presence is controversial because the relation of generic *E. coli* with a pathogen may vary on the environmental setting (i.e., samples type, climate and landscape, among others) (Ceuppens et al., 2015). In some cases, the number of samples is insufficient to establish a significant relationship between the indicator and the pathogen presence (Sagoo et al., 2003). Thus, the relation of *E. coli* with pathogen presence is quite complex, whether *E. coli* may serve as a suitable index microorganism or not depends on the pathogen, the climate and seasonality, the geographic region, the sample type and the presence of animal reservoirs.

However, the use of generic *E. coli* as an indicator of faecal contamination of produce has been a common practice (Byappanahalli et al., 2006; Mukherjee et al.,

2007; Holvoet et al., 2014a). Although generic *E. coli* can form stable populations in temperate soil and water environments (Byappanahalli et al., 2006; Ishii et al., 2006), its survival is indicative of conditions favourable for the survival and persistence of pathogenic *E. coli* and *Salmonella* spp. (Natvig et al., 2002; Park et al., 2013).

Some studies have confirmed the potential usefulness of generic *E. coli* as an index organism for the presence of *Salmonella* spp. (Natvig et al., 2002; Wilkes et al., 2009; Park et al., 2013, Holvoet et al., 2014a) and *E. coli* O157: H7 (Ogden et al., 2001). A recent study performed across farms in various countries with variable climate and agro-technical management practices showed that elevated *E. coli* numbers had moderate to good predictive value on the presence of *Salmonella* spp. and STEC but not for *Campylobacter* (Ceuppens et al. 2015). This is in accordance with previously published research (Carter et al., 1987). However, no defined number of generic *E. coli* in leafy greens or water was shown to serve as a threshold value to distinguish between safe and unsafe produce or irrigation water (Ceuppens et al., 2015). On the other hand, some studies have not found any significant correlation between *E. coli* and pathogens such as *Salmonella*, *Campylobacter*, *E. coli* O157, other STEC, *Cryptosporidium*, *Giardia*, *Aeromonas hydrophilia*, *Legionella pneumophila* (Ahmed et al., 2010), *Campylobacter* (Savill et al., 2001) or *Salmonella* spp. (Economou et al., 2012). Therefore, the identification of risk factors and sources for the contamination of leafy greens with generic *E. coli* can be a good strategy to improve the control of foodborne illnesses related to these commodities (Park et al., 2014).

Within the current legislation in the European Union, *E. coli* is included as process hygiene criterion. Process hygiene criteria for fresh-cut fruits and vegetables are based on the Commission Regulation EU Nº 1441/2007 (OJEU L322/12-29, 7

December 2007). These criteria for *E. coli* are shown in **Table 1.5** and the limits refer to each sample unit tested,

Table 1.5. *E. coli* limits as process hygiene criteria for RTE fruit and vegetables.

Food category	Sampling plan		Limits		Stage where criteria applies	Action in case of unsatisfactory results
	n	*c*	*m*	*M*		
RTE fruits & vegetables	5	2	100 CFU/g	1000 CFU/g	Manufacturing process	Improvements in production hygiene, selection of raw materials

6.2.2 Other indicator microorganisms

- **Coliforms**

Coli-aerogenes (coliform) bacteria have been used worldwide for the verification of the absence of enteric pathogens, or faecal contamination, in drinking water and foods for a century (EPA, 2011). Total coliforms are aerobic or facultatively anaerobic, Gram-negative, non-spore-forming bacteria that produce gas and acid upon lactose fermentation within 48 h at 35 °C. Faecal coliforms are a subset of coliforms that also ferment lactose at 44 °C (Leclerc et al., 2001). They belong to the family *Enterobacteriaceae* and include *E. coli* and various members of the genera *Enterobacter*, *Klebsiella* and *Citrobacter* (DiSalvio, 1997). Some of these species are encountered in one frequently unique habitat. However, a majority of these organisms are ubiquitous, residing in soil, surface water, intestinal tract of man and animals, estuarine fish, and molluscs, surface of leafy plants, insects, rodents, etc. (WHO, 2001).

Coliforms either total or faecal are common choices of indicator organisms and have been the most frequently studied indicators as they were included in drinking water regulations. They are still recognized as acceptable indicators especially in disinfection processes (WHO, 2003; Wu et al., 2011). Some studies have used this

group to assess the microbial overall quality of leafy greens or water samples (De Quadros-Rodríguez et al., 2013). However, alternative indicators to coliforms have been proposed because this group presents some limitations such as: large number of environmental species (Edberg et al., 2000; WHO, 2001), lower environmental resistance than protozoa, differential transport characteristics from viruses (Savichtcheva & Okabe, 2006; Wu et al., 2011), non-faecal source (Scott et al., 2002), possibility of regrowth and greater weakness to the disinfection process (Hurst, 2002), or low correlation with the presence of pathogens and low sensitivity of detection methods (Horman et al., 2004). Despite of its limitations it is interesting that in some studies total coliforms and faecal coliforms showed a greater correlation with pathogen than the faecal indicators *E. coli* and enterococci (Wu et al., 2011). Payment & Locas (2010) also found that the non-enteric indicators, total coliforms and aerobic endospores, were more frequently observed in virus-positive samples in groundwater as compared to *E. coli* and enterococci. On the contrary, coliforms have considered as an unreliable indicator of faecal contamination as some authors have reported waterborne diseases outbreaks in water meeting the total coliform regulations (Payment et al., 1997).

In conclusion, as soon as the coliform test came into widespread acceptance, complications with its use an interpretation began to emerge. One concern was the discovery that a variety of microorganism that read positive in the coliform test was not of a faecal origin. As a result, the test method has evolved continually to become more specific (i.e. faecal coliform test which selects for coliforms of faecal origin by using higher incubation temperature (Odonkor & Ampofo, 2013).

- **Enterobacteriaceae**

The family *Enterobacteriaceae* encompasses approximately 20 genera, including *E. coli* and all members of the coliform group; in addition it includes foodborne pathogens *Salmonella*, *Shigella*, and *Yersinia*. The family was originally proposed as an indicator alternative to the coliform group because testing for the entire family would be more inclusive for the pathogenic bacteria (Rajwar et al., 2015). The *Enterobacteriaceae* may be superior to coliforms as indicators of sanitation GMPs because they have collectively greater resistance to the environment than the coliforms (Mossel et al., 1978). The determining factor separating coliforms from *Enterobacteriaceae* is the ability of coliform to ferment lactose, while the *Enterobacteriaceae* family ferments glucose. The determination of *Enterobacteriaceae* to assess and subsequently improve the hygiene of production and the quality of foods and feeds was introduced many years ago (Mossel et al., 1995). Several studies have tested *Enterobacteriaceae* as hygiene or overall quality indicator of leafy greens. They reported that all the samples were generally contaminated with variable counts of this indicator (Leifert et al., 2008; Valentin-Bon et al., 2008; Abadias et al., 2012; Cardamone et al., 2015). Nevertheless, a big part of the *Enterobacteriaceae* family are not pathogens, many of them are commensals of the mammalian gastrointestinal tract and they can be also found in abundance in almost any moist environment, notably soil, water and plants, and can be found among the flora associated with growing vegetables making that the result of finding high counts of this group have to be interpreted carefully and high counts of these microorganism do not necessarily compromise produce safety.

- **Enterococci**

The enterococci were first integrated into the functional group of bacteria known as “faecal streptococci” but now largely belong to the genus *Enterococcus* which was

formed by the splitting of *Streptococcus faecalis* and *Streptococcus faecium*, along with less important streptococci, from the genus Streptococcus (Schleifer & Kilpper-Balz, 1984). In addition, other Enterococcus species and some species of Streptococcus (namely *S. bovis*, and *S. equinus* are included in this group (Muruleedhara et al., 2012). Enterococci can be regarded as indicators of faecal pollution since they have a number of advantages as indicators over total coliforms and even *E. coli* (Geldreich et al., 1997). Some of these advantages are that they generally do not grow in the environment, are highly resistant to drying and survive longer (Muruleedhara et al., 2012).

Summarizing, a new paradigm is evolving regarding indicator and index microorganism, the suitability of one type of indicator or another depends on the question being asked (Yates, 2007). The possible correlation with pathogens relies also in the amount of available data since higher correlations are more frequently reported for systems/study sites with higher number of positive samples for pathogens (Wu et al., 2011; Ceuppens et al., 2015). Holvoet et al. (2014a) reported high correlation (0.79-0.92) between *E. coli*, total coliforms and enterococci in irrigation water indicated that it is not necessary to enumerate all hygiene indicators as they were strongly correlated to each other. This study also reported that *E. coli* is preferable as indicator of unsanitary conditions in comparison to coliforms due to its faecal origin (Holvoet et al., 2014a).

It can be concluded that much of the controversy with regards to indicators and pathogen correlation is the result of studies with insufficient data for assessing such correlations (Wu et al., 2011). Moreover, it has been demonstrated that one indicator might not represent the relative abundance of all pathogenic bacteria, viruses and protozoa. Thus, combined application of indicator could lead to more comprehensive results about faecal contamination and its association with pathogenic microorganisms.

7. Significance of quantitative microbial risk assessment and predictive modelling

Quantitative microbial risk assessment (QMRA) is a systematic quantitative assessment process to estimate the health risks or illness rates of human exposure to particular pathogens. The approach combines dose response information for the infectious agent with information on the distribution of exposures routes and predictive modelling (Haas et al., 1999). It is traditionally defined as consisting of four components: hazard identification, dose-response assessment, exposure assessment, and risk characterization, which when considered together provide an expression of microbial risk (CAC, 1999). For the development of quantitative exposure assessment of a specified food and foodborne pathogen, statistical distributions of microbial concentrations are used as input values (such as bacterial concentrations in raw materials subjected to further process) and/or as output values (predicted contamination in foods after processing and/or storage) (Hoornstra & Notermans, 2001; Crépet et al., 2007). Thus, the estimation of microbial risk inherently contains variability and uncertainty so that simulation methods such as Monte Carlo are commonly used in QMRA (FAO/WHO, 2009a). The accuracy of QMRAs is improved by the availability of measurements related to the behaviour of hazards in specific foods and the development of mathematical models able to predict exposure due to contamination at the various stages along the farm-to-consumption chain (McKellar et al., 2014).

Predictive modelling is a potential alternative approach that can overcome the limitation of challenge testing (Baranyi & Roberts, 1995). This is possible when key environmental factors that affect survival and growth can be identified. It must develop models that relate these factors to the behaviour of the organism under conditions of interest. In addition, models can enable researchers to make genuine comparisons

between treatments so long as parameter values obtained from various treatments according to the model are consistent with practical realities (Ongeng et al., 2014). Furthermore, mathematical models are valuable in undertaking quantitative microbiological assessment of vegetables at pre-harvest (Franz et al., 2010).

A number of studies have focused in applying mathematical modelling and quantitative microbial risk assessments (QMRA) to predict pathogen survival and assess the risk of leafy greens contamination at different levels in the farm to fork continuum. For example, within the most recent published research a QMRA for spinach associated with *E. coli* O157:H7 (Danyluk & Schaffner, 2011), leafy green vegetables in salad associated with *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* (Franz et al., 2010), lettuce associated with *L. monocytogenes* (Ding et al., 2013) and *E. coli* O157:H7 (Ottoson et al., 2011). Recently, a QMRA study was also 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). One limitation of development of QMRAs for leafy vegetables including pre-harvest level is that all of them are hampered by the lack of models that can simulate pathogen behaviour in the field (McKellar et al., 2014). Recent investigations conducted with leafy greens at pre-harvest have yielded kinetic data that could be used to develop suitable quantitative approaches in order to predict the fate of enteric pathogens following a contamination event (Erickson et al., 2010b; Moyne et al., 2011; Bezanson et al., 2012). In a recent study by McKellar et al. (2014) the available data was used to develop quantitative approaches and models describing the fate of the *E. coli* O157:H7 species in the lettuce field to be included in future QMRA studies.

QMRA can be a useful strategy for growers in relation to water and soil management practices (i.e. manure application) because it can be applied to establish

the links between concentrations of pathogenic microorganisms in agricultural water (Stine et al., 2005; Hamilton et al., 2006; Mota et al., 2009; Seidu et al., 2013; Barker, 2014) and soil (Franz et al., 2008a) and the probability of illness. A study performed by O'Toole et al. (2010) showed how to translate an outcome target to performance targets for water treatment, and irrigation and farming practices. This research highlighted that microbial risk assessment can be used in a regulatory framework to guide food producers to the appropriate risk management interventions based on a combination of barriers in the chain from irrigated fresh produce to consumer (O'Toole et al., 2010). In addition, in 2007 the EFSA established the EFSA Scientific Network on microbiological risk assessment (MRA Network). The MRA hold biannual meetings aimed to facilitate a scientific cooperation framework by the coordination of activities, the exchange of information, the development and implementation of joint projects and the exchange of expertise and best practices. The MRA activities are focused on filling data gaps and setting priorities for data collections. Currently, 22 European Union Member States and two observer countries (Switzerland and Norway) are members of the MRA Network (EFSA, 2014b).

It could be concluded that QMRA, using scenario analysis and predictive microbiology, constitutes a useful approach on the ongoing efforts to manage food risks (Bassett et al., 2012). Additionally, it can be a valuable tool for enhancing food safety by evaluating the effects of intervention measures in food production processes (Vercammen et al., 2013). However, there are still some limitations regarding the interpretation of QMRA outcomes. This is because risk assessments are often confronted with variability or uncertainty of data sets (Vasquez et al., 2014). Thus, risk assessors have to deal with lack of information and need to use surrogate data or assumptions. Therefore, as QMRA outcomes rely partly on assumptions, results should

be interpreted as an indication of the level or degree of safety and not as absolute values (De Keuckelaere et al., 2015). Still, the outcomes and estimation provided by QMRA can be used to guide the risk management in preventing and controlling contamination, as well as in the identification of areas where further research or data collection are needed.

Chapter II: Objectives

The general objective of this thesis is to evaluate the main microbial risk factors affecting the safety of leafy greens, specifically baby spinach during primary production and further processing.

The specific objectives of this research can be divided into three sections:

1. Primary production level (pre-harvest level)

Contamination of leafy greens may occur at any step in the farm to consumer chain (growing, harvest, processing, wholesale, transport, retailing and handling at home). However, recent publications have highlighted pre-harvest level as the most probable origin of potential contamination. Agricultural practices (irrigation water, fertilizer application), environmental factors and weather conditions (rainfall, floods, temperature and solar radiation), among others may influence the microbial contamination of leafy greens. Therefore, baseline studies focused on evaluating how these factors affect microbial safety of produce are still needed. In this section, the objectives were:

- To describe the distribution of indicator microorganisms (generic E. *coli*, total coliforms, *Enterobacteriaceae* and *Enterococcus*) and the prevalence of foodborne pathogens (*L. monocytogenes*, pathogenic *E. coli* and *Salmonella* spp.) during the production of baby spinach grown in the Southeast of Spain.
- To evaluate the effect of weather and agricultural practices on the contamination of baby spinach with generic *E. coli* and foodborne pathogens at pre-harvest level.
- To evaluate the impact of extreme water related events (flooding) in the microbial safety of leafy greens at field level.

- To develop a QMEM that allows to assess the impact of weather conditions (e.g. solar radiation, cultivation season, rainfall, flooding) and field agricultural practices (e.g. irrigation method, irrigation water source) on the microbial safety of leafy greens.

2. Processing level

Due to the absence of an inactivation step before consumption, products labelled as ready-to-eat (RTE) are potential sources of human pathogens. It is clear that the microbial safety of the raw materials together with the hygienic status of the processing environment (contact surfaces, washing steps and equipment), are significant factors for the microbial safety of RTE**.** Generally, the assessment of microbial safety relies on end produce testing to evaluate compliance or not with the implemented microbiological standards and guidelines; however it seems that systematic analysis for pathogens in end product or in the production environment is not likely enough to guarantee the safety of RTE products. Therefore, there is still a need for the identification of suitable sampling points and methodologies that facilitate the detection of contaminated produce and assure the testing of the entire production. In this section, the main objectives were:

- To assess the microbiological risk associated with key operations units involved in the RTE leafy green processing chain.
- To evaluate the suitability of generic *E. coli* as a hygiene criterion at processing of leafy greens that could be considered for validation and verification of Good Hygiene Practices.

3. Consumption level

The increase in foodborne outbreaks have highlighted the importance of evaluating the risk associated with the fresh produce production chain though exposure assessment, a key step in risk assessment. In order to evaluate the potential risk associated with fresh produce consumption there is a need of standardizing food consumption data (i.e. frequency of consumption, portion sizes) together with handling practices. This data can be used to perform further investigations for microbiological and/or chemical exposure assessment. The objectives of this section were:

- To evaluate the suitability of existing data related to the consumption of fresh fruits and vegetables in two European countries: Belgium and Spain.
- To obtain (through a survey) consumption data for fresh produce consumed raw or minimally processed in Belgium and Spain and to provide suitable data for further exposure assessments.
- To study handling practices of fresh produce by Belgian and Spanish consumers.

Chapter III.

Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain

1. Introduction

Contamination of leafy greens with foodborne pathogens may occur at any step in the farm to consumer chain (growing, harvest, processing, wholesale storage, transportation, retailing and handling at home) from environmental, animal or human sources (FAO/WHO, 2008; FDA, 2009a; EFSA, 2013). Recent publications highlighted several pre-harvest sources as the most probable origins of potential contamination including: contaminated water, soil amendments and faecal contamination from wildlife (Pachepsky et al., 2011; Doyle & Erickson, 2012; Ceuppens et al., 2014; Holvoet et al., 2014a). Little data on the microbial quality and safety of baby leaves during pre-harvest is available generating the need for more studies on specific agricultural practices and microbiological risks.

The most common etiologic agents associated with produce outbreaks are *Escherichia coli* O157:H7 and *Salmonella* (Mootian et al., 2009). Recently, European Food Safety Authority (EFSA) highlighted *Salmonella* spp. and leafy greens eaten raw as salads as one of the five top raking food/pathogen combinations most often linked to human cases originating from Food of Non-Animal Origin (FoNAO) in the EU (EFSA, 2013). These microorganisms can persist in the environment for long periods of time, and they may spread to and contaminate distant locations. Recent studies have shown that bacterial survival in the field is significantly influenced by environmental and weather conditions. In the US, precipitation has been identified as a predictor of spinach contamination with generic *E. coli*, indicating that the probability of contamination increases with an increase in the amount of rain over the past month (Park et al., 2014). Seasonal differences in the microbial concentrations on fresh produce have been also reported showing higher counts of indicators in the fall (September, October and

November) compared to spring and winter (Ailes et al., 2008). In Belgium, Holvoet et al. (2014a) found a direct correlation between indicator bacteria and pathogens in irrigation water with temperature and precipitation. These findings highlight the utility of weather databases to obtain hourly and daily weather information to predict contamination and demonstrate that environment and weather factors should be considered together to develop Good Agricultural Practices (GAPs) guidelines and measures to reduce produce contamination (Park et al., 2014). There is a clear increase in cases of salmonellosis when ambient temperatures increase (D'Souza et al., 2004; Kovats et al., 2005; Fleury et al., 2006; Semenza et al., 2012). For many years, there is great interest in determining the most common causes of the increase in produce-associated outbreaks in the summertime (FDA, 2001). However, the mechanisms underlying the observed seasonality in foodborne disease are not fully understood, but are likely to involve a complex interplay of multiple factors (Liu et al., 2013). *Salmonella* spp. is susceptible to climatic variables as it is vulnerable to sunlight and drying out, but their survival can be promoted at higher temperatures (McMichael et al., 2006). In fact, van Pelt et al., (2004) reported that above a 6 ºC threshold, the risk of *Salmonella* infection increased in several European countries.

Due to the prohibitive cost of pathogen detection, many researchers use microbial indicators to characterize microbial contamination in the environment of field cultivation and fresh produce (Park et al, 2013). Although generic *E. coli* can form stable populations in temperate soil and water environments, its presence is indicative of conditions favourable for survival and persistence of pathogenic *E. coli* and *Salmonella* spp. (Park et al., 2013). Its presence on produce indicates faecal contamination and thus possible presence of pathogens carried in the intestinal tract of animals (Adams & Moss, 2000). This microorganism has been recognized as a good indicator for the

presence of faecal contamination and a good index indicator for the presence of *Salmonella enterica* serovar Typhimurium (Natvig et al., 2002), *Salmonella* spp. (Park et al., 2013) and *E. coli* O157:H7 (Ogden et al., 2001).

To effectively reduce the prevalence of foodborne pathogens in baby leaves at the pre-harvest level, both the contamination routes and meteorological factors affecting pathogens' survivability should be considered (Park et al., 2014). The aim of the present study was to describe, for the first time, the distribution of indicator microorganisms (generic *E. coli*, total coliforms, *Enterobacteriaceae* and *Enterococcus*) and the prevalence of foodborne pathogens (*Listeria monocytogenes,* Enterohaemorrhagic *E. coli* and *Salmonella* spp.) with respect to the potential risk factors in the production of baby spinach grown in the Southeast of Spain. The Southeast area of Spain is considered the garden of Europe, as it is a leading European horticultural area. The impact of weather factors on the contamination of baby spinach with generic *E. coli* and foodborne pathogens at the pre-harvest level was also evaluated. Moreover, the relationship between the distribution of indicator microorganisms and the presence of foodborne pathogens in a sample was established.

2. Materials & Methods

2.1 Production farms

Three of the biggest Spanish leafy green growers agreed to participate in this study. All farms were located in the southeast of Spain between Murcia and Almeria. The specific location was kept confidential to protect the identity of the farmers. The dimension of the farms ranged between 2 and 4 ha. Irrigation water on these farms was

from ponds; overhead sprinkler irrigation was used for irrigation. Irrigation was usually carried out every day in the morning.

2.2 Sampling plan

The study took place between November 2011 and April 2013. A systematic longitudinal sampling plan was developed to identify potential risk factors for microbial contamination in the production of baby spinach. The sampling sites were selected based on the literature review of potential risk factors that contribute to microbiological contamination, particularly in leafy greens (Pachepsky et al., 2011; Olaimat & Holley, 2012; Park et al., 2012). For each selected farmer, the sampling plan included sample collection during 3 production cycles distributed throughout a growing season, which excludes summer (from May to August) because there is no summer production in this area. The duration of the production cycle, considered from the day of seeding until the day of harvest, varied depending on the part of the season and it was on average 8 and 5 weeks in winter and spring, respectively. During each complete production cycle, 4 visits were carried out and 4 codes were used for sampling-time identification: T1= at planting, T2= 2 weeks before harvest, T3= one week before harvest and T4= at harvest. The sampling was carried out over the three individual production cycles during the growing season for the three farms with a total of 540 samples collected during the study: 27 samples of manure, 27 samples of seeds, 120 samples of soil, 150 samples of water, 108 samples of baby spinach, 81 samples of surfaces and 27 samples of worker' hands.

2.3 Sampling methodology

The protocol previously described by Holvoet et al., (2014a) was followed. For solid samples (soil, seeds, manure and baby spinach), 9 samples of approximately 100 g

each were randomly collected. In the case of soil and fresh produce, samples were taken from different locations in the field following a zig-zag pattern started from a randomly selected side of the field. Soil samples were taken at the surface (0-5 cm depth) within a 20 cm diameter around each sampled plant using a spade previously disinfected with 70% ethyl alcohol. Manure and seed samples were taken at random sites at the company storehouse. Once in the laboratory, the solid samples (100 g each) were randomly pooled into 3 samples (25 g each). In the case of water, samples were collected from ponds and at the irrigation head (outlet of the irrigation system to the produce). Four litre samples were collected into sterile bottles according to ISO 19459:2006 (ISO, 2006). For sampling of surfaces (conveyor belt, blade and boxes) and worker's hands, sterile swabs were used for swabbing of 50 cm^2 of surface area and both hands, respectively. The swab was immersed in 5 mL buffered peptone water and transported to the lab. Microbial analyses were conducted within 2-14 h from the time of sample collection.

2.4 Microbial analysis

2.4.1 Indicator microorganisms

Counts of indicator microorganisms were monitored as previously described (Holvoet et al., 2014a). *E. coli,* total coliforms and *Enterococcus* were enumerated in 100 mL water samples using cellulose nitrate membrane filters (0.45µM diameter, Microsart®, Sartorius, Spain). *Enterobacteriaceae* were only determined in surface and worker's hands samples. Chromocult Agar (AES Chemunex, France, Europe) was used for the enumeration of *E. coli* and total coliforms after incubation for 24 h at 37 °C in solid, water and surface samples. *Enterococcus* were incubated on Slanetz and Bartley medium (Oxoid, UK, Europe) for 44 h at 37 °C. Then, filters were transferred to bile-aesculine-azide agar (Sigma Chemical, MO, US) for 2 h at 44 ºC. *Enterobacteriaceae*

were enumerated using Violet Red Bile Glucose (Oxoid) after incubation for 24 h at 37 °C. The detection limits were 100 CFU/g in case of solid samples, 50 CFU/surface in case of surfaces and workers' hands, and 1 CFU/100 mL in case of water samples. The surface unit corresponded to the surface of both worker's hands or the area of 50 cm^2 of a food contact surface.

2.4.2 Pathogenic microorganisms

Presence or absence of VTEC (*E. coli* O157:H7 and other verocytotoxin producing *E. coli*: O26, O103, O111, O145) and *Salmonella* spp., were determined in solid and water samples (n=144) as previously described (Desroche et al., 2009; Holvoet et al., 2014a). Solid samples (25 g each) were homogenized for 1 min in 225 mL of BPW (AES Chemunex) and incubated for 18 ± 2 h at 37 °C for enrichment. Water samples (1 L each) were filtered and the filters were incubated in 100 mL BPW at 37 °C for 18 - 20 h for enrichment. Fifty µL of each enriched sample were used to extract and purify the bacterial DNA using a commercial extraction kit (Extraction Pack Food for *Salmonella*, STEC, EHEC, *E. coli* O157:H7 and *Listeria* detection (Pall®, WA, US). Samples were analysed using the validated (Beutin et al., 2009; Delbeke et al., 2015b) method of GeneDisc® Rapid Microbiology System (Pall® Corporation, WA, US). The selected GeneDisc Plates allowed the detection of a range of microorganisms including pathogenic *E. coli* O157, STEC and *Salmonella* spp. In the case of a positive PCR signal for pathogen presence by the GeneDisc® multiplex PCR, isolation and confirmation of colonies was attempted. Before isolation, 1 mL of frozen (30% glycerol) enriched samples was subjected to second non-selective enrichment in 10 mL of BPW (AES Chemunex) at 37 ºC for 18-24 h. For the confirmation of *Salmonella* spp. positive samples, the ISO 16140:2003 method (Anonymous, 2003b) was used for further isolation of presumptive *Salmonella* spp. colonies.

2.5 Meteorological parameters

Weather data for ambient temperature, precipitation, solar radiation and relative humidity (RH) were obtained during the sampling period from the nearby climatic stations at 'Pozohiguera' (37º 30' 13,86" N, 1º 41' 38,07" W), 'Torre Pacheco' (37º 44' 51,81,N 0º 59' 14,02" W) and 'Balsa-Pintada' (37º 44' 53,89" N, 1º 7' 45,14" W), located within 10 km of the sampled fields. For each location and sampling day, the climate data were obtained from the nearby climate station. The climatological database of Sistema de Información Agraria de Murcia (SIAM) was used (SIAM, 2014).

2.6 Statistical analysis

Non-zero microbial loads were log-transformed (base-10) and stored along with zero counts (for samples with undetected contamination) in an Excel spread sheet (Microsoft Excel, 2010). For calculation and graphical presentation of the median and interquartile range (IQR) of microbial counts only positive samples (i.e., with numbers above the detection limit) were included. IBM SPSS Statistics 19 was used for statistical analysis. Except when stated otherwise, *P* values below0.05 were considered statistically significant. Shapiro-Wilk test was performed to assess the normality of the data ($P>0.05$). Mann Whitney U and Kruskal-Wallis tests were used to respectively determine the difference between the positive counts (non-zero microbial loads) of the indicators with respect to presence/absence of pathogens and to define differences in counts between producers and farm management practices. To compare prevalence of samples positive for indicator across different types of samples, the chi-square (χ^2) test was used. Bivariate correlation analysis (Spearman´s rank) to assess correlations between individual explanatory variables when one or both of the explanatory variables were continuous was used. Correction for multiple comparisons was not performed

because the goal of the study was to identify all potentially important statistical associations for baby spinach contamination in the study area in order to build a foundation for future research.

3. Results and Discussion

3.1 Indicator microorganisms

Positive samples for generic *E. coli* were found in soil (18/120), irrigation water (78/150) and baby spinach (6/108) (**Figure 3.1**). In soil, one of the most frequent sources of contamination is the use of organic amendments for fertilization. Transmission of foodborne pathogens from amended soil with contaminated manure from cattle, sheep, pig and chicken to leafy greens has been reported (Solomon et al., 2002; Islam et al., 2004a,b; Ongeng et al., 2011b; Oliveira et al., 2012). Survival for prolonged periods in manure and amended soil has also been demonstrated (Himathongkham et al., 1999; Franz et al., 2007, 2008a). In the present study, soil samples were analysed at two different stages representing soil amendment with the organic matter: (i) at planting and (ii) during cultivation (1 and 2 weeks before harvest) and at harvest. Significant differences (χ^2 test, P=0.001) were found between the *E. coli* prevalence in soil at planting (n=39) and during cultivation (n=81) with a percentage of positives of 38.5% versus 3.8%, respectively. This could be explained by the bacterial decay after fertilization as described by Lang et al. (2007) who reported an exponential decay of 1 log 100 g^{-1} per 30 days. Mukherjee et al. (2004) reported that farms that used manure or compost aged for less than 12 months had a prevalence of *E. coli* 19 times greater than conventional samples. Accordingly, Park et al. (2013) reported that the odds of *E. coli* contamination on spinach were almost 13 times lower when the time from the last manure spreading was >200 days (Odds Ratio=0.08). Based on our results

and literature (Park et al., 2013; USDA, 2000), current recommendations on the applications of manure at least 90 days prior to harvesting can reduce pre-harvest contamination of baby leaves with generic *E. coli* as indicators of faecal contamination.

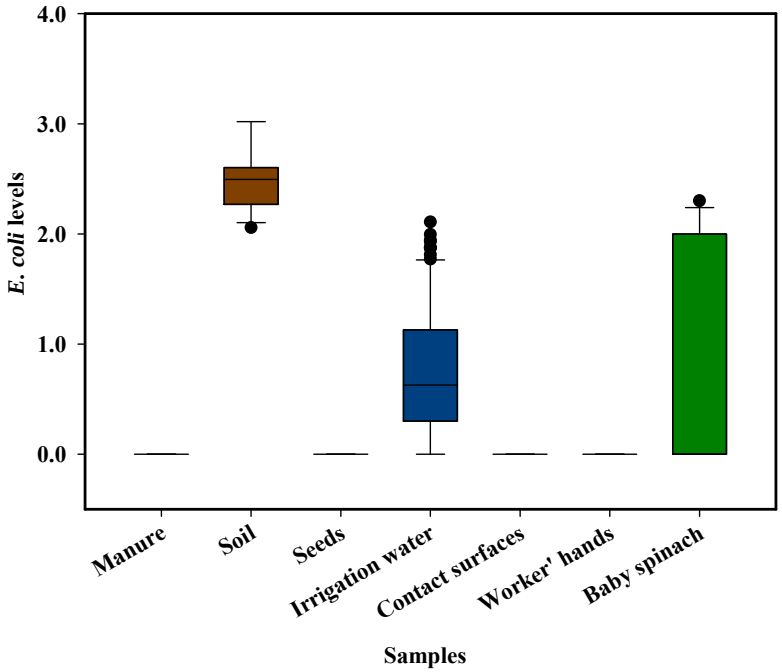

Figure 3.1. Boxplots representing *E. coli* counts (log CFU/g, log CFU/100 mL or log CFU/cm^2) for positive environmental and produce samples. In this study positive samples are define as samples contaminated above detection limit. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). For sample types without a boxplot, the horizontal line at zero *E. coli* level indicates complete absence of positive samples.

Irrigation water was obtained from ponds, which collected water from rain and from the Tajo Segura water transfer. All the producers applied spray irrigation, which increased the probability of contamination. Park et al. (2013) highlighted the use of pond water for irrigation as a strong predictor of spinach contamination. In the present study, water samples (n=150) showed significantly higher *E. coli* prevalence (χ^2 test, $P<0.001$) and counts (Mann-Whitney test $P<0.05$) in water from ponds (70.8% prevalence with the median count in positive samples of 0.9 log CFU/100 mL (IQR 0.8

to 1) compared to water from irrigation heads (34.6% prevalence with the median count in positive samples of 0.4 log CFU/100 mL (IQR 0.3 to 0.6). Higher prevalence of *E. coli* in irrigation water for whole lettuce has been reported (de Quadros Rodrigues et al., 2014; Holvoet et al., 2014a). Our results show the high prevalence of *E. coli* in agricultural water from ponds and confirm irrigation water as one of the most important routes of faecal contamination from their reservoirs to leafy greens (Pachepsky et al., 2011; Park et al., 2012). The limits established for *E. coli* in irrigation water in the Brazilian and Spanish legislation are 2 x 10^2 and 10^2 CFU/100 mL, respectively (de Quadros Rodrigues et al., 2014; RD 1620, 2007) whereas other microbial quality standards established maximum *E. coli* counts up to 10^3 CFU/100 mL (Pachepsky et al., 2011). These levels are much higher than the average values found in the water samples tested in this study. The guidelines for GAPs recommend growers to treat the irrigation water to avoid any potential contamination when the contamination of the water is above the acceptable limit thresholds (FAO/WHO, 2008; FDA, 2009a). Different water management practices were applied on each farm to treat the irrigation water and reduce water contamination. Briefly, Producer 1 applied potassium permanganate (2 mg/L $KMnO_4$) while Producer 2 used filtration and Producer 3 irrigated with untreated water. Potassium permanganate is a strong oxidizing agent highly toxic for bacteria (Tucker & Boyd, 1977). Filtration is a well-known water treatment able to reduce the microbial load (Gómez et al., 2006). We observed that the three enrolled spinach producers had similar median counts of *E. coli* in contaminated irrigation water samples (from ponds and irrigation heads) (**Figure 3.2**). However, significant differences (χ^2 test, $P<0.001$) were found regarding the prevalence between treated and untreated water at both the pond and the irrigation head. Treated water from irrigation head showed a prevalence of 46.1% while untreated water showed a prevalence of 100%. These results support the recommendation for water treatment to ensure the safety of the irrigation water as

important intervention strategy to reduce the potential for contamination of fresh produce (Gil et al., 2015).

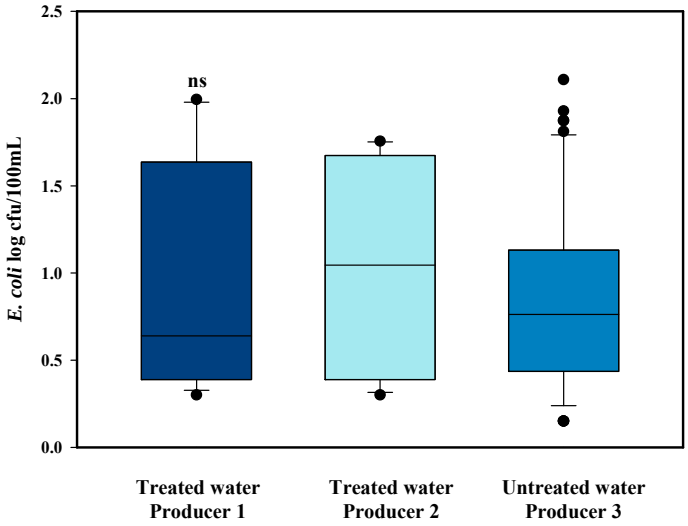

Figure 3.2. *E. coli* counts (log CFU/100 mL) in positive water samples obtained from different water sources for each baby spinach producer. In this study positive samples are define as samples contaminated above detection limit. Significant differences between producers were determined by Mann-Whitney test ($P<0.05$) and are represented with different letters and 'ns' means no significant. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile).

E. coli prevalence on contact surfaces (conveyor belt, blade and boxes) and workers' hands was monitored at harvest. Spinach was mechanically harvest and sampling included the conveyor belts and blades of the harvesters. However, none of the tested samples was positive for generic *E. coli*, which may be explained by good hygiene practices. Park et al. (2013) have stated that there is a lack of epidemiological studies of hygiene practices at the pre-harvest level. They reported an association between a composite variable that included (among other factors) the workers' hygiene practices (in terms of the use of portable toilets and washing stations in the field and training of staff/temporary workers to use portable toilets) and produce contamination

prevalence at the pre-harvest level. Nevertheless, positive samples for workers' hands have been reported, suggesting that personal hygiene should be considered as a potential factor for controlling microbial contamination of produce (de Quadros Rodrigues et al., 2014).

From all tested produce samples (n=108), 5.6 % were positive for generic *E. coli* with the median microbial count of 2.2 log CFU/g (IQR 2.0 to 2.3). The prevalence observed in the current study is similar than that reported by Park et al. (2013) who observed that 6.6% of spinach samples were positive for generic *E. coli* in farms located in Colorado and Texas (US). In lettuce, de Quadros Rodrigues et al. (2014) reported higher *E. coli* counts, up to 3.6 log CFU/g, in lettuce produced in Brazil under conventional and organic farming. Additionally, Oliveira et al. (2010) reported that *E. coli* was found in 12.5% of whole lettuce produced under conventional farming. These differences in the prevalence of generic *E. coli* could be due to specific characteristics of the crop but also due to the small sample numbers and different detection limits applied in these studies. For example, in Park et al (2014) the detection limit was 4 CFU/mL of the plated dilution (which corresponds to the detection limit of 4 CFU/g of spinach) compared to the detection limit of 100 CFU/g of spinach in the current study. Thus, these types of studies should be considered, at best, a snapshot of the microbiology of the product in question.

Apart from generic *E. coli*, total coliforms and other microbial groups were also monitored in the selected samples to evaluate their value as indicator microorganisms although several authors have already reported a poor association (Gayeon et al., 2013; Pahl et al., 2013). In the present study, manure, soil, seeds, irrigation water and baby spinach samples were positive for coliforms. The values obtained were in accordance with data reported for total coliforms in environmental and lettuce samples (de Quadros

Rodrigues et al., 2014) although this information is very difficult to interpret due to the limitations of total coliforms as an indicator of faecal contamination. In the case of coliforms on contact surfaces (blades, conveyor belts and boxes) 64.2% of samples were positive with median microbial count of 2.7 CFU/surface (IQR 2.4 to 3.1), while no coliforms were found on workers' hands (**Table 3.1**). As far as we know, there are no surface specifications for coliforms, although general microbial target values of <2.5 CFU/cm^2 of surface area have been suggested (Moore & Griffith, 2002). The samples tested in our study were around this limit, and in agreement with counts in surface samples (ca. 2.1±0.8 CFU/cm^2) in a study carried out in Brazil (de Quadros Rodrigues et al., 2014).

The levels of *Enterobacteriaceae* were also monitored as a hygiene indicator of contact surfaces and workers' hands as they have been defined as effective tools to assess the improvements in GAPs and manufacturing practices (van Schothorst & Oosterom, 1984). All the tested samples were positive for *Enterobacteriaceae*, showing median microbial counts of 4.2 log CFU/surface (IQR 2.4 to 3.1), and 3.4 log CFU/surface (IQR 3.3 to 3.5) for contact surfaces and workers' hands, respectively (**Table 3.1**).

Enterobacteriaceae is a large and diverse group and although they may be useful indicators for overall GAPs they are not necessarily indicators of faecal contamination. Therefore, their relevance at primary production level should be interpreted carefully because of their ubiquitous distribution (Lues & Van Tonder, 2007).

Table 3.1. Microbial counts of coliforms, *Enterobacteriaceae* and *Enterococcus* in manure, soil, seeds and baby spinach (log CFU/g), irrigation water (log CFU/100 mL) as well as contact surfaces and workers' hands (log CFU/surface). Results show median value and interquartile range (IQR) for positive samples only (i.e., samples contaminated above the detection limit). NA: not analysed. T1= Planting day, T2=2

weeks before harvest. T3=1 week before harvest, T4=harvest day. Irrigation water results are for combined pond and irrigation head samples.

Sample	Coliforms		*Enterobacteriaceae*		*Enterococcus*	
	Median (Prevalence)	IQR	Median (Prevalence)	IQR	Median (Prevalence)	IQR
T1						
Manure	5.1 (11/27)	4.6-5.2	NA	-	NA	-
Soil	3.8 (39/39)	3.4-4.6	NA	-	NA	-
Seeds	5.3 (27/27)	5.3-6.2	NA	-	NA	-
T2						
Soil	3.0 (27/27)	2.5-3.6	NA	-	NA	-
Irrigation water	2.2 (51/51)	1.9-3.2	NA	-	0.9 (34/51)	0.4-1.3
Baby spinach	3.2 (27/27)	2.9-3.6	NA	-	NA	-
T3						
Soil	3.3 (27/27)	2.9-3.6	NA	-	NA	-
Irrigation water	2.2 (51/51)	3.1-1.9	NA	-	1.1 (24/51)	0.4-1.4
Baby spinach	3.6 (27/27)	3.3-4.3	NA	-	NA	-
T4						
Soil	3.5 (27/27)	3.3-4.0	NA	-	NA	-
Irrigation water	3.1 (48/48)	2.0-3.2	NA	-	1.1 (32/48)	0.6-1.5
Conveyor belt	2.4 (16/27)	2.2-2.7	4.3 (15/15)	3.0-4.6	NA	-
Blade	3.0 (18/27)	2.5-3.3	4.1 (15/15)	3.2-4.4	NA	-
Boxes	3.1 (18/27)	2.7-3.1	4.1 (15/15)	3.8-4.8	NA	-
Worker' hands	<1.7 (0/27)	-	3.4 (15/15)	3.2-3.5	NA	
Baby spinach	3.6(54/54)	3.2-3.9	NA		NA	

The prevalence of *Enterococcus* spp. in irrigation water obtained from ponds was 60%, with the median count of 1.1 log CFU/100 mL (IQR 1.3 to 0.3) for the positive samples. As observed for *E. coli* levels, significant differences (χ^2 test, P=0.001) were found for the prevalence of *Enterococcus* spp. between the irrigation water samples obtained from ponds (70.8%) and irrigation heads (16.0%) (Data not shown). *Enterococcus* spp. has been traditionally used as an indicator for faecal contamination. It has been reported that the bacterial load of stored water may increase during the storage in an open well and a part of the microbial contamination may originate from faecal contamination by birds and mammals that have access to water storage (Sazakli et al., 2007; Schets et al., 2010).

In the present study, only a weak negative correlation (Spearman's rho coefficient = 0.4) between *E. coli* and total coliforms was found in irrigation water, indicating a low association between them (data not shown). Some publications have already focused on the correlations between different indicators such as *E. coli*, coliforms and *Enterococcus* spp. in water, although conclusions are often contradictory (Economou et al., 2012; Holvoet et al., 2014a). Gayeon et al. (2013) and Pahl et al. (2013) reported that no correlations were found between faecal indicators on fresh produce and those found in irrigation water, suggesting that the use of a single microbial indicator for water is of limited value for predicting the safety of water for irrigation of produce. Results obtained in the present study agree with these references regarding the weak negative correlation between microbial indicators for baby spinach and irrigation water (**Figure 3.3A**). However, a positive correlation was found between total coliforms in soil and baby spinach (**Figure 3.3B**). The Spearman rank correlation coefficient obtained 0.6 ($p<0.01$), which suggests that part of the variation in the count of coliforms on spinach could be explained with coliform counts in soil. These observations might support previous research that indicates that bacteria contaminating the soil can reach the plant through different mechanisms, such as soil splashing (Girardin et al., 2005; Monaghan & Hutchison, 2012).

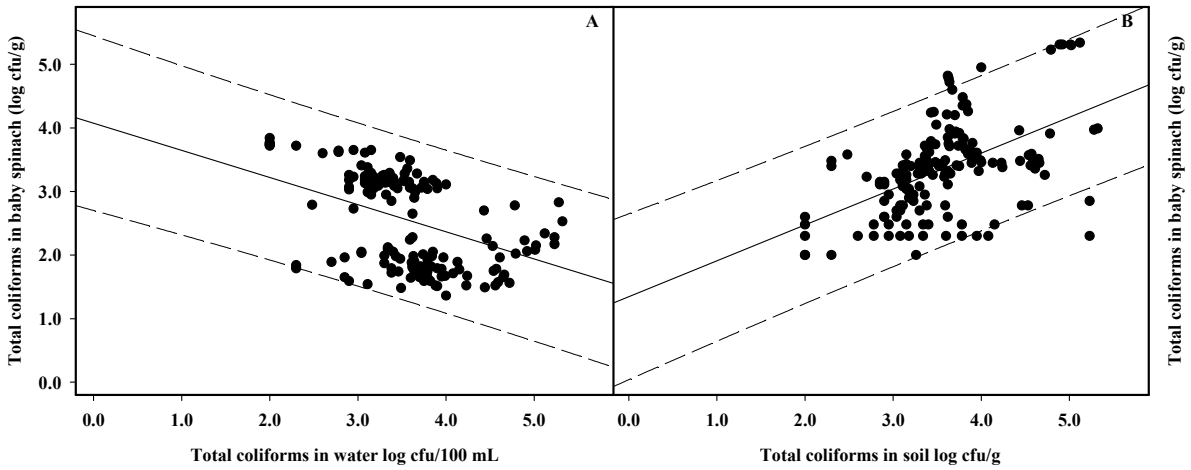

Figure 3.3. Scatter plots showing the relationship (A) between total coliforms in water samples (log CFU/100 mL) and total coliforms in baby spinach (log CFU/g) and (B) between total coliforms in soil (log CFU/g) and total coliforms in baby spinach (log CFU/g). Confidence intervals at 95% (broken lines) and central regression lines are represented.

3.2 Impact of climatic conditions on indicator microorganisms

The influence of climatic conditions on the generic *E. coli* levels in irrigation water was assessed using the mean climatic parameters obtained for the week before sampling and grouped into intervals 5-10^{o}C, 10-15^{o}C and 15-20^{o}C. We did not observe any significant impact of temperature on the *E. coli* levels in irrigation water (**Figure 3.4A**). Holvoet et al. (2014a) reported that the highest levels of *E. coli* were observed at the time of the year when the outside temperature and the water temperature were the highest. They also found a significant correlation between precipitation and *E. coli* contamination of the irrigation water. However, this tendency was not confirmed in our study (data not shown). Huge differences in rainfall and irrigation sources between Belgium and our study area in Spain might explain these differences.

Positive *E. coli* samples (6 out of 108 tested) of baby spinach were all detected when the temperature was in the highest range (15-20 ºC), while the rest of the samples were all negative (**Figure 3.4B**). This result could be partially explained by higher

microbial counts at higher temperatures, which made the detection of contamination more likely. This is in agreement with our recent study which indicated a quadratic relationship between generic *E. coli* count and the average maximum daily temperature over the 9 days prior to sampling with the highest bacterial count at around 24 °C (Park et al., 2015). Combinations of environmental factors have been described to influence the frequency and transmission of foodborne pathogens and subsequently to impact the risk of produce contamination (Strawn et al., 2013a). Precipitation has been also highlighted as another relevant climatic factor directly related with the higher probability of spinach contamination (Park et al., 2014). However, in the present study, neither precipitation nor RH seemed to have influenced *E. coli* prevalence in baby spinach (data not shown). This could be due to the low number of positive samples and the low rainfall during the two years that the study was conducted, which made it difficult to establish correlations. This also cautions about the need for careful generalization of findings about meteorological risk factors to untested distant locations.

When temperatures increased, there was a tendency to increase total coliforms in soil and baby spinach, while the levels decreased with temperature in irrigation water (**Figure 3.5**). When coliforms levels were studied as a function of RH, no significant differences were found in soil (**Figure 3.6A**).

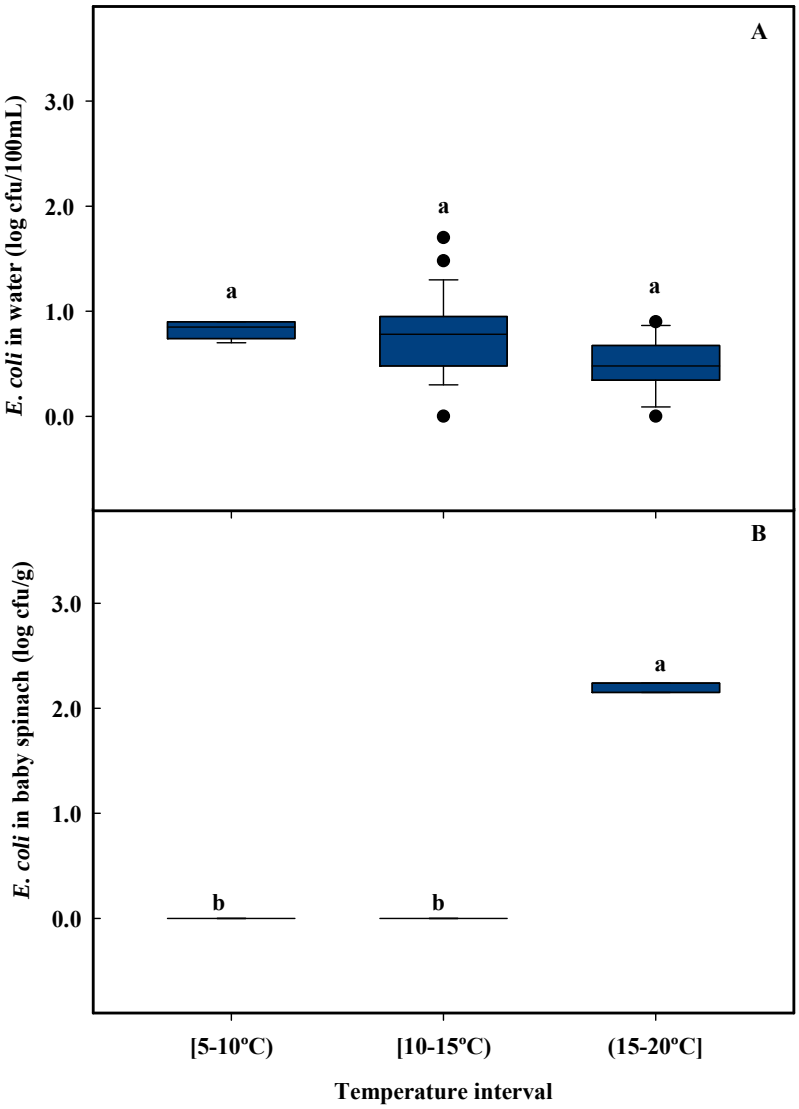

Figure 3.4. Boxplot representing **(A)** E. coli counts (log CFU/100 ml) in positive water samples and **(B)** *E. coli* counts (log CFU/g) in positive baby spinach as a function of the mean ambient temperature during the week before sample collection (ºC). In this study positive samples are define as samples contaminated above detection limit. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). For temperature ranges without a boxplot, the horizontal line at zero *E. coli* level indicates complete absence of positive samples. Significant differences were determined by Mann-Whitney test ($P < 0.05$) and are represented with different letters.

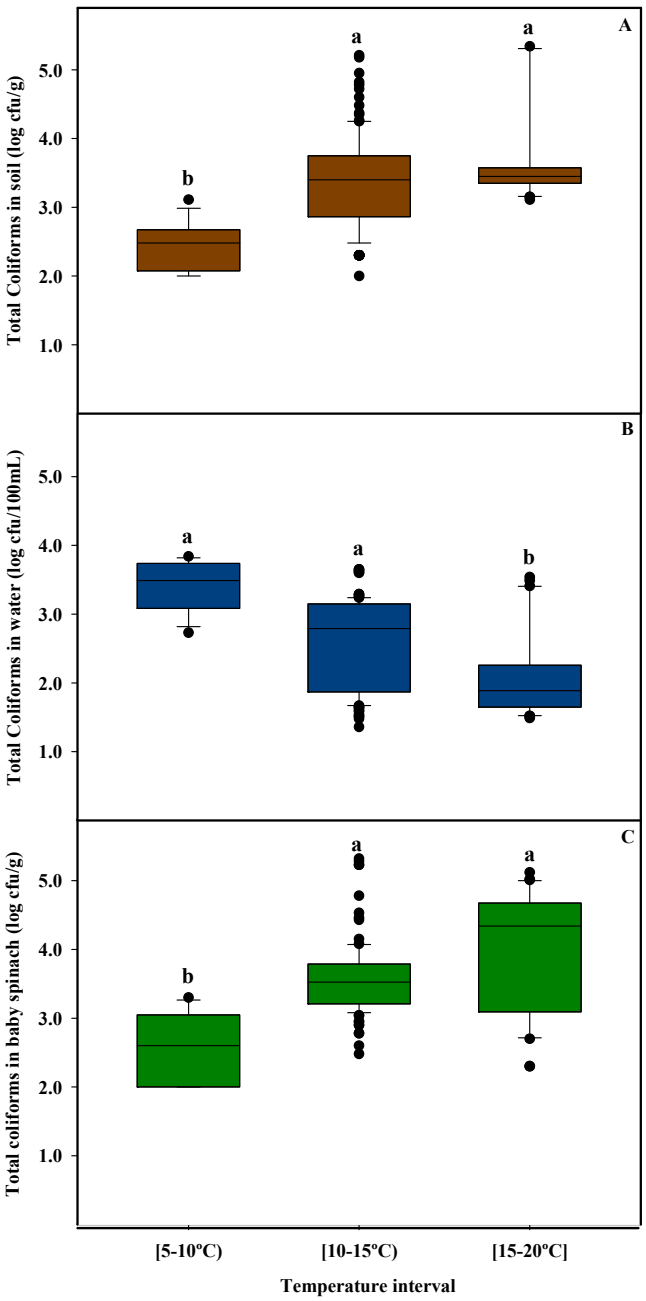

Figure 3.5. Boxplot representing total coliforms counts in soil (log CFU/g) **(A)**, water (log CFU/100 mL) **(B)**, and baby spinach (log CFU/g) **(C)** as a function of the mean ambient temperature during the week before sample collection (ºC). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). Significant differences were determined by Mann-Whitney test ($P < 0.05$) and are represented with different letters.

In the case of baby spinach the significant differences (Mann-Whitney test, *P*=0.007) in coliform counts were only found between the lowest and the highest RH which were correlated with the lowest and highest coliform counts, respectively (**Figure 3.6B**). To the best of our knowledge, there are no other available data regarding the impact of these climatic parameters (precipitation and RH) on coliforms.

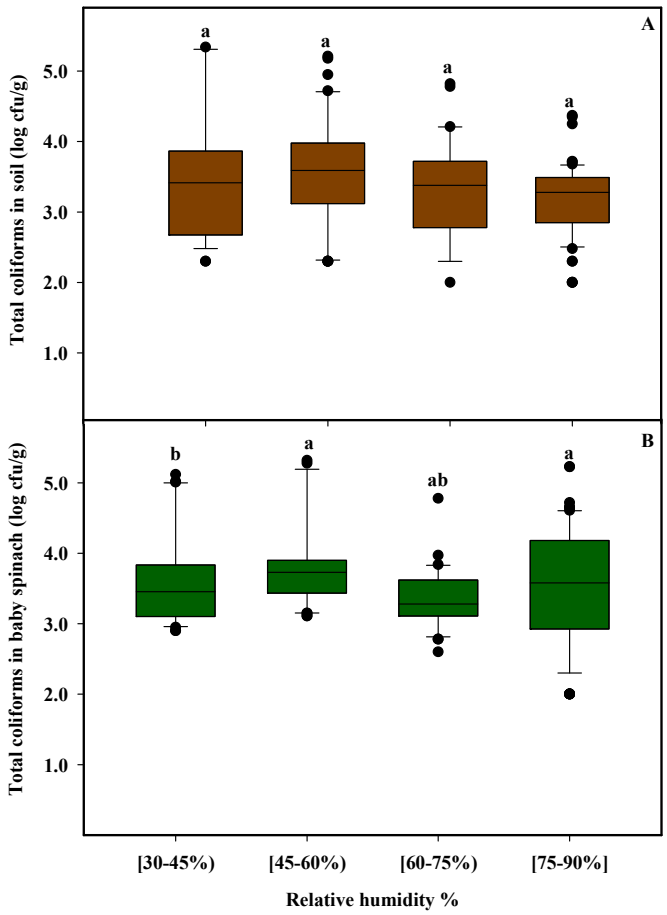

Figure 3.6. Boxplot representing total coliforms counts in soil (log CFU/g) **(A)** and baby spinach (log cfu/g) **(B)** as a function of the mean ambient relative humidity during the week before sample collection. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). Significant differences were determined by Mann-Whitney test ($P < 0.05$) and are represented with different letters.

3.3 Pathogens

A total of 144 samples of manure, soil, seeds, irrigation water and baby spinach were analysed for pathogens. *L. monocytogenes* was analysed only in baby spinach, but no positive samples were detected (data not shown). Pathogenic *E. coli* was not found in any of the tested samples (**Table 3.2**). These negative results could be partially explained by the small number of samples and sample size.

Table 3.2. Pathogen microorganisms (*Salmonella* and pathogenic *E. coli*) in manure, seeds, soil, irrigation water and baby spinach.

Sample	*E. coli* O157:H7		*E. coli* O26, O103, O111 & O145		*Salmonella*	
	Genedisc®	Confirmed*	Genedisc®	Confirmed*	Genedisc®	Confirmed*
Manure	0/9	--	0/9	--	2/9	1/2
Soil	0/40	--	0/40	--	2/40	0/2
Seeds	0/9	--	0/9	--	0/9	--
Irrigation water	0/50	--	0/50	--	1/50	1/1
Baby spinach	0/36	--	0/36	--	2/36	0/2

*Samples were confirmed by culture isolation and/or PCR using different targets.

Salmonella spp. was detected by multiplex RT-PCR analysis in 7 samples including manure (2/9), soil (2/40), irrigation water (1/50) and baby spinach (2/36). However, only two of the RT-PCR positives were confirmed by culture, one corresponding to manure and another to irrigation water (**Table 3.2**). Recent studies already highlighted the identification of presumptive positive colonies in environmental and fresh produce samples as a challenge, probably due to the presence of a wide range of indigenous competing microbiota on selective agars (Delbeke et al., 2015b). Positive samples for *Salmonella* spp. presence in manure have been previously reported but they have been traditionally associated with the production of organic lettuce and the use of inefficient composting process for the organic fertilizers (de Quadros Rodrigues et al., 2014). However, in this study, sampling was carried out in conventional farms, where

composted manure was always applied, suggesting failures in the composting process (Lung et al., 2000; Jiang et al., 2002). The relevance of the implementation of a well-controlled fertilizer program has been already included in most of the GAPs guidelines (FAO/WHO, 2008; FDA, 2009a; EFSA, 2014a), which should represent a method for growers to avoid potential soil contamination through fertilizers (Johannessen et al., 2005). Survival of foodborne pathogens such as *Salmonella* spp. and *E. coli* O157:H7 in soil amended with contaminated compost has been shown to be from 7 to up to 23 weeks (Islam et al., 2004a; Oliveira et al., 2012). Based on these results, it is recommended to guarantee manure composting because the duration of the growth cycle of baby spinach, which varies, depending on the season, between 8 weeks in winter and 5 weeks in spring will not be enough to assure the decline of these pathogens before harvest.

Prevalence of presumptive *Salmonella* spp. in irrigation water was very low (2%) (**Table 3.2**), similar to that previously described (de Quadros Rodrigues et al., 2014; Holvoet et al., 2014a). Although the small number of tested samples limits generalizations, the presence of foodborne pathogens in the irrigation water and the prevalence of generic *E. coli* observed in these samples are in agreement with previous studies, which highlighted irrigation water as a potential risk factor for introduction of pathogens in the primary production of leafy greens (Pachepsky et al., 2011; Park et al., 2012; Ceuppens et al. 2014).

Even if generalizations are difficult to make from such a limited sample, an underlying trend was that higher *E. coli* counts could be associated with the RT-PCR detected presence of *Salmonella* spp. in soil, irrigation water and baby spinach (**Figure 3.7**). This is in agreement with previous studies, which reported correlations between the prevalence of pathogens and selected hygiene criteria (Holvoet et al., 2014a; Castro-

Ibáñez et al., 2015a). This finding may support the hypothesis that considers *E. coli* as a good microbial indicator of faecal contamination that had a predictive value for pathogen presence.

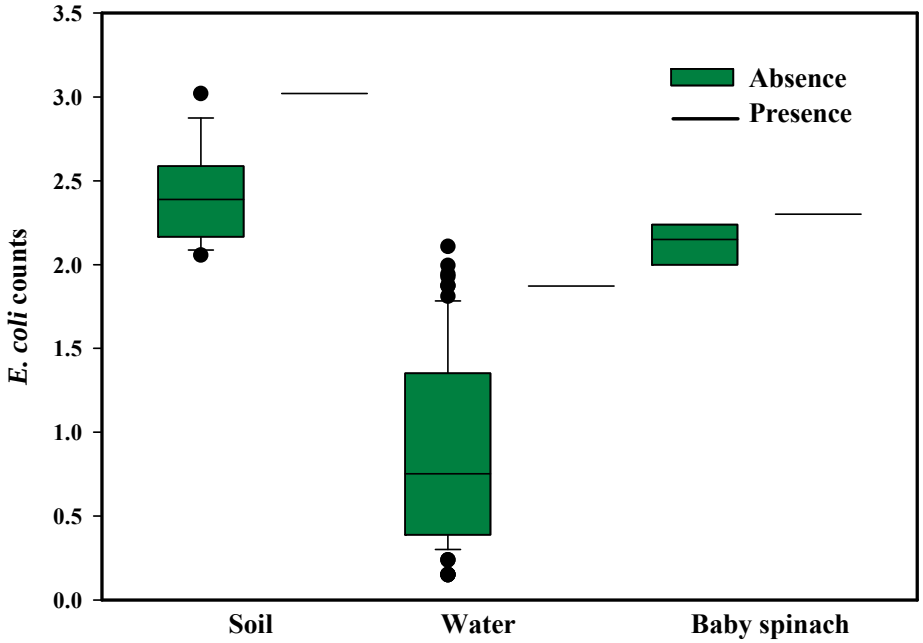

Figure 3.7. Graph representing *E. coli* counts (log CFU/g or log CFU/100 mL) in *E. coli* positive samples separated into those with absence or presence of *Salmonella* where *Salmonella* presence was defined as positive signals by GeneDisc® PCR The horizontal lines show *E. coli* median values of one or two *Salmonella* spp. positives per sample type.

This study has a few limitations. The three enrolled producers were among the largest baby spinach producers in the study area. This was important to assure that the results of this study are relevant to a large segment of the produced baby spinach in this area of Spain. However, because producers of different sizes may have different management practices caution is needed in extrapolating the results to all producers in the study area. Notably, this study evaluated three growing cycles for each of the three enrolled producers, which provided microbial quality data for the whole growing season. However, because growing seasons differ in terms of the weather patterns, including in occurrence of extreme events (e.g., floods), caution is needed in

extrapolating the results of this study to the future. In the current study, the number of tested samples was relatively small (144) and the prevalence of samples contaminated with indicator microorganisms was likely underestimated due to relatively high limits of detection and the related expected false negative results for samples contaminated at low levels.

4. Conclusions

Based on the literature, soil and irrigation water have been highlighted as two of the most important factors affecting the microbial quality of baby spinach. The use of water treatments has been described as a good intervention strategy to reduce microbial contamination of irrigation water and maintain a constant water quality all year around, which might help to reduce the uncertainty associated with the safety of fresh produce. The results obtained showed that water treatment could be recommended as a mitigation option to reduce microbial risk of fresh produce. Ambient temperature seemed to affect the levels of indicator microorganisms in baby spinach as all *E. coli* positive samples were detected at the highest observed temperature range. However, caution is needed in extrapolating the results of this study mostly due to the limited number of tested samples. Results regarding the correlation between *E. coli* levels and pathogen presence suggest *E. coli* as a potential hygiene criterion at primary production of leafy greens.

Chapter IV.

Microbial safety considerations of flooding in primary production of leafy greens: A case study

1. Introduction

Climate change affects the frequency, intensity and duration of extreme water-related weather events such as excessive rainfall and flooding (Semenza & Menne, 2009; Pachauri et al., 2012). Flooding accounts for about 40 % of all natural disasters that occurred worldwide 20 years ago (French et al., 1989). Recently, Cann et al. (2013) have reported that out of all outbreaks associated with extreme water-related weather events, heavy rainfall and flooding were by far the most common climate change effects. The increasing intensity of heavy rainfall is projected to make extreme river floods even more frequent in some areas, especially in central, northern and north-eastern Europe (IPCC, 2012).

Consequences of climate change have been identified as having potential for increasing bacterial contamination of food and water (Tirado et al., 2010). A study conducted during flooding in the US in 2001 identified an increased incidence of gastrointestinal illness due to flooding (Salvato et al., 2003). Flooding may have multiple food safety consequences, particularly if the agricultural land is adjacent to livestock farms and industrial and residential areas (Miraglia et al., 2009). These events may lead to the contamination of soil, irrigation water and produce with pathogens from the contaminated floodplain sources (Confalonieri et al., 2007). Flooding may have an impact on the persistence and patterns of occurrence of bacteria and it affects the ecology of microbes (Tirado et al., 2010). In a study carried out by Orozco et al. (2008), the presence of *E. coli* and *Salmonella Newport* was demonstrated in tomato samples during and after a flooding event. Consequently, fresh produce grown in contaminated land after flooding has been recognized as a potential vehicle for transmission of pathogenic microorganisms (CAC, 2003; EFSA 2013). The consequence of outbreaks

associated with fresh produce result in considerable economic losses to farmers, distributors and the food industry (Golberg et al., 2011). Recently, a guide has been established to assess growers on the intervention strategies to mitigate these risks (CDC, 2011). This guide specified that after a flood event, health authorities should follow a risk assessment measure to determine the safe use of previously flooded outdoor areas. In general, product contamination is reduced with longer intervals between flooding and the harvest of the plant (FDA, 2011). Using experimental data, several studies have attempted to develop recommended intervals between field contamination and harvest but vary significant in their designation of a safe time period (Doyle & Erickson, 2008). Most of the information related to potential sources of pre-harvest contamination has been acquired from experimental studies in the laboratory or field trials in which they have demonstrated, after artificial inoculation, the persistence of foodborne pathogens for different periods of time (Tomás-Callejas et al., 2011). However, to evaluate the microbial contamination risk after flooding is a challenge because of difficulties in developing an adequate experimental design. This is mostly because of the sporadic nature of these events, which make it difficult to repeat the sampling in a specific setting. Thus, attempting to establish a safe interval between the flood and the harvest to avoid microbial risks is challenging. The objective of the study was to evaluate the effects of a flood event, flood plain and climatic parameters on microbial contamination of leafy greens grown in the flood plains. The relationships between indicator and pathogenic microorganisms were also established.

2. Materials & Methods

2.1 Sampling area

At the end of September 2012, an extreme event of heavy rainfall occurred in the Southeast of Spain, which caused flooding in most of the adjacent lands and growing fields. This area is characterized by a dry climate with an average rainfall of 40.7 mm in September during the last 12 years. However, in this water-related event, the rainfall was 84 mm in 24 hours. This region has been subjected to several flooding events (**Table 4.1**).

Table 4.1. Flooding events recorded in the south-east area of Spain in the last 4 years. Data represent the monthly average rainfall (mm) between 2009 and 2012 and the rainfall (mm) per month and per 24 h of specific years.

Dates	Monthly average rainfall (2009-2012)	Rainfall/month (specific year)	Rainfall/24 h (specific year)
March	26.4±31.3	107.4 (2009)	67.2 (2009)
May	37.0±33.6	115.6 (2009)	48.7 (2009)
August	14.5±28.1	92.2 (2010)	59.7 (2010)
September	40.7±41.8	137.5 (2009)	96.8 (2009)
		88.5 (2012)	84.0 (2012)

2.2 Climatic parameters

Climatic data were obtained from the nearby Barranda climatic station (38º 35’ 6’’ N, -1º 40’47 994’’ W) and Purias climatic station (37º 33’ 53,95’’ N, -1º 41’ 49,2’’ W) located within 10 km of the fields, using the local climatological database (SIAM,

2014). Relevant climate parameters, such as temperature, rainfall and solar radiation were collected daily during the sampling period.

2.3 Sampling plan

Four growing fields of iceberg lettuce (*Lactuca sativa* L.) affected by flooding were selected and sampled for this study (**Figure 4.1**). **Field 1** (38º 35' 6'' N, -1º 40' 47,994'' W)**,** located in Lorca (Murcia, Spain) with a surface area of 3.5 ha without a watercourse in the surroundings; **Field 2** (37º 33' 53,95'' N, -1º 41' 49,2'' W), located in Lorca (Murcia, Spain) with a surface area of 6.2 ha and a watercourse at 250 m; **Field 3** (38º 2' 38,62'' N, 1º 58' 35,52'' W), located in Caravaca (Murcia, Spain), with a surface area of 7.6 ha and a watercourse at 316 m; and **Field 4** (38º 2' 31,45'' N, 1º 58' 34,52'' W), located in Caravaca (Murcia, Spain) with a surface area of 4.8 ha and a watercourse at 210 m.

Figure 4.1. Map of the farm layout showing the location of the four growing fields.

Sampling was done approximately in weeks 1, 3, 5 and 7 after the flooding. Soil in this growing area was sand clayed organically manured. Commercial harvest of the lettuce was carried out after 10 weeks of the flooding. At each sampling time, 9 samples of soil and 9 whole head iceberg lettuces were randomly collected from different locations in the field following a zig-zag pattern which started from one of the sides of the field. Soil samples were taken at the surface (0-5 cm depth) within a 20 cm diameter by using a spade. Nine samples of irrigation water were taken from the irrigation systems at each sampling time, except for Field 1, where because of the severity of the flood event, the systems were not available and samples were taken from the water reservoir. Samples of lettuce in Field 1 were also not available because of the severity of the flood event. All samples were stored and transported in the dark at 4 °C to the lab (max. 40 km) for further handling (cutting/pooling). The sampling methodology used in this study followed the protocol previously described by Holvoet et al. (2014a). Summarizing, 9 soil samples (100 g each) and 9 lettuce samples from the edible leaves were randomly pooled by 3 in the lab. Microbial analyses were conducted within 2-14 h.

2.4 Microbial analysis

2.4.1 Indicator microorganisms

Soil and lettuce samples of 25 g each were homogenized in a 1:10 dilution of sterile 0.1% buffered peptone water (BPW; AES Chemunex, BioMérieux SA, France). Water samples (2 L each) were collected into sterile bottles according to ISO 19458 (ISO, 2006). Serial dilutions of samples were performed and plated on the appropriate culture media.

Coliforms, *E. coli* and *Enterococcus* were enumerated in 100 mL water samples using cellulose nitrate membrane filters (0,45µM diameter, Microsart®, Sartorius, Madrid, Spain), while coliforms and *E. coli,* were quantified in soil and lettuce samples. Chromocult Agar (AES Chemunex), a selective chromogenic medium, was used for the enumeration of *E. coli* and total coliforms after incubation for 24 h at 37 °C. Enumeration of coliforms and *E. coli* in water samples were performed according to ISO 9308-1 (ISO, 2000a) with the exception that the Tergitol 7 medium was replaced by Chromocult Agar. *Enterococcus* was enumerated according to ISO 7899-2 (ISO, 2000b). Briefly, filters were incubated on Slanetz and Bartley medium (Oxoid, Hampshire, UK) for 44 h at 37 °C. Then, filters were transferred to bile-aesculine-azide agar (Sigma Chemical, St. Louis, MO) for 2h at 44 ºC.

2.4.2 Pathogenic microorganisms

Presence or absence of *Salmonella* spp., VTEC (*E.coli* O157:H7 and other verocytotoxin producing *E. coli*, O26, O103, O111, O145) and *Listeria monocytogenes*, were determined in all the samples as previously described (Holvoet et al., 2014a; Desroche et al., 2009). Samples of 25 g of soil and lettuce were homogenized for 1 min in 225 ml of BPW (AES Chemunex) and incubated for 18 ± 2 h at 37 °C for enrichment. In this case, the 9 samples of each type of sample (soil and lettuce) were further pooled in one sample. Water samples (1 L each) were filtered and the filters were incubated in 100 ml BPW at 37 °C for 18 - 20 h for enrichment. Then, 50 µL of all enriched samples were used to extract and purify the bacterial DNA using a commercial extraction kit (Extraction Pack Food for *Salmonella*, STEC, EHEC, O157:H7 and *Listeria* detection, Pall®, France). Part of the enriched samples was also kept at -80 ºC for further analysis. Once the DNA was extracted, samples were analysed using the validated method of GeneDisc® Rapid Microbiology System (GeneSystems, France).

Commercially available GeneDisc® plates were used for the screening in parallel of specific gene sequences of human pathogenic verotoxin producing *E. coli* virulence factors (*stx1*, *stx2*, *eae*), *E. coli* O157:H7 ($rfbE_{O157}$ and $fliC_{H7}$) and *Salmonella* spp. specific genes (*iroB*), while also including inhibition control and negative control (Beutin et al., 2009). In the case of a positive PCR signal for pathogen presence by the GeneDisc® multiplex PCR, isolation and confirmation of colonies was attempted. Before isolation, 1 mL of frozen (30% glycerol) enriched samples was subjected to second non-selective enrichment in 10 mL of BPW (AES Chemunex) at 37 ºC for 18-24 h. For the confirmation of *Salmonella* spp. positive samples, the ISO 16140:2003 method (Anonymous, 2003b) was used for further isolation of presumptive *Salmonella* spp. colonies. Briefly, samples were subjected to a selective enrichment in BPW supplemented with Ibisa specific supplement (ISS) (AES Chemunex) at 41 ºC for 16 - 20 hours. Then, 10 µL of the enriched broth was plated on IBISA medium (AES Chemunex) and incubated at 37 ºC for 24 h to detect typical green colonies with esterase activity. Positive colonies were confirmed in *Salmonella* selective media, Salmonella Agar Plate (ASAP) and Xylose-Lysine-Desoxycholate Agar (XLD) (AES Chemunex). Confirmation of *E. coli* O157:H7 positive samples was carried out using Inmunomagnetic (Dynabeads®) separation and then plating in two selective media: MacConkey Sorbitol agar (CT-SMAC, Oxoid) and a ChromIDO157 (Biomérieux, France). Positive colonies were then confirmed using Oxoid *E. coli* O157 Latex. For the rest of VTEC strains, the confirmation was carried out as previously described by Holvoet et al. (2014a). In the case of positive *L. monocytogenes*, samples were enriched for *Listeria* species in Half-Fraser Broth (Oxoid) at 30° C for 24 h. After selective enrichment, cultures were then plated consecutively on ALOA® (Biomérieux, France) for isolation of presumptive colonies of *L. monocytogenes* and ALOA confirmation® (Biomérieux) for confirmation. Confirmed positive samples for pathogens were

submitted to the Spanish Type Culture Collection (CECT, Valencia, Spain) for serotyping.

2.5 Experimental design and statistical analysis

Microbial loads were log-transformed and introduced in Excel spreadsheet along with time (raw microbial data). Results were compiled and graphs were made using Sigma Plot 12.0 Systat Software, Inc. (Addilink Software Cientifico, S.L. Barcelona). The regression analysis was implemented using SigmaPlot nonlinear regression analysis by means of the exponential decay equation [$f=y_0+a\cdot e^{(-bt)}$]. Confidence intervals on the estimated microbial data were also computed. The confidence intervals define the uncertainty range on the estimated regression parameters and were established at 95%. IBM SPSS statistics 19 was used for statistical analysis. Mann-Whitney U test was used to determine the difference between the raw data of the indicators and the presence of pathogens.

3. Results and Discussion

3.1 Indicator microorganisms

Irrigation water samples collected 1 week after the flood event showed levels of both, coliforms and *E. coli*, close to 5 and 4 log CFU/100 mL, respectively, indicating a high contamination of the environment due to flooding (**Figure 4.1**). In the surrounding area of flooding, there were several farms, mostly swine farms, which may have contributed to the contamination of the flooded areas. These results confirmed the risk of irrigation water contamination of farms in flooded areas next to livestock farms (CDC, 2011). Coliform counts drastically declined 3 weeks after the flood event but remained with counts of 1-2 log units /100 mL (**Figure 4.2**).

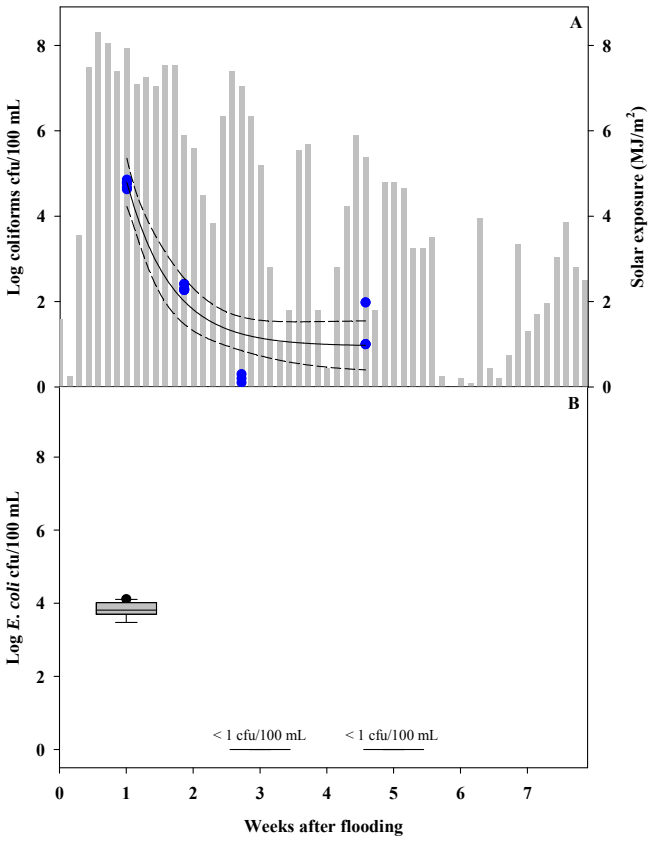

Figure 4.2. (A) Changes in coliforms (log CFU/100mL) in water (dots) and solar exposure (bars) after the flooding event. Solid line represents best-fitted equation and dot lines are confident bands generated by non-linear regression analysis. **(B)** Boxplot of *E. coli* (log CFU/100mL) in water after flooding event. Bottom and top of the box are the 25th and 75th percentile and the ends of the whiskers are the minimum and maximum of all the data. Dots represent outlier values.

Predicted kinetic parameters and statistics associated with the non-linear regression analysis are presented in **Table 4.2.** The regression analysis was carried out to determine if changes in microbial counts observed after the flooding event can be modelled and satisfactorily fitted using a decay equation. This analysis resulted in an exponential decay equation with a coefficient of determination (R^2) and the Standard Error (S_{yx}) of 0.86 and 0.65, respectively. The observed microbial decrease was associated to the insolation conditions observed during two weeks after the flood event, which were very high, with values between 6 and 8 MJ/m^2. Whitman et al., (2004)

reported that during sunny days (average insolation of 1.7 MJ/m^2), *E. coli* counts decreased exponentially with day length and exposure to insolation, suggesting that solar inactivation is an important mechanism for the natural reduction of bacteria.

Table 4.2. Estimated parameters (y_0, a, b), R^2 and standard error (S_{yx}) for coliforms in water, soil and lettuce. Exponential decay equation (Log Coliforms (CFU/g or CFU/100mL) = $y_0 + a \bullet e^{-b \bullet t}$) was obtained by non-linear regression analysis where t=time in weeks after flooding event.

Sample	y_0	a	b	R^2	Standard Error (S_{yx})
Water	0.96±0.29	17.40±6.65	1.51±0.41	0.87	0.66
Soil	3.31±0.15	3.98±1.45	0.90±0.39	0.72	0.42
Lettuce	3.08±0.25	6.39±1.78	0.82±0.27	0.81	0.52

Therefore, the significant reduction of coliforms was probably due to the climatic conditions of this time of the year that are characterized by high solar radiation and temperature (**Figure 4.2**). No detection of *E. coli* was possible 3 and 5 weeks after flooding with a detection limit of 10 CFU/ 100 mL)

When soil samples were analysed, a similar tendency to that found in irrigation water was observed. High levels of coliforms, close to 6 log CFU/gr, were found 1 week after of the flood event and they drastically declined after 3 weeks (**Figure 4.3**). In fact, values obtained 3 weeks after flooding were within the normal levels for total coliforms in soil (3-4 log units) previously described for this area (Selma et al., 2007). *E. coli* levels in soil were particularly high in samples taken 1 week after flooding but they were below the detection limit (2 log cfu/g) after 3 and 5 weeks (**Figure 4.3**).

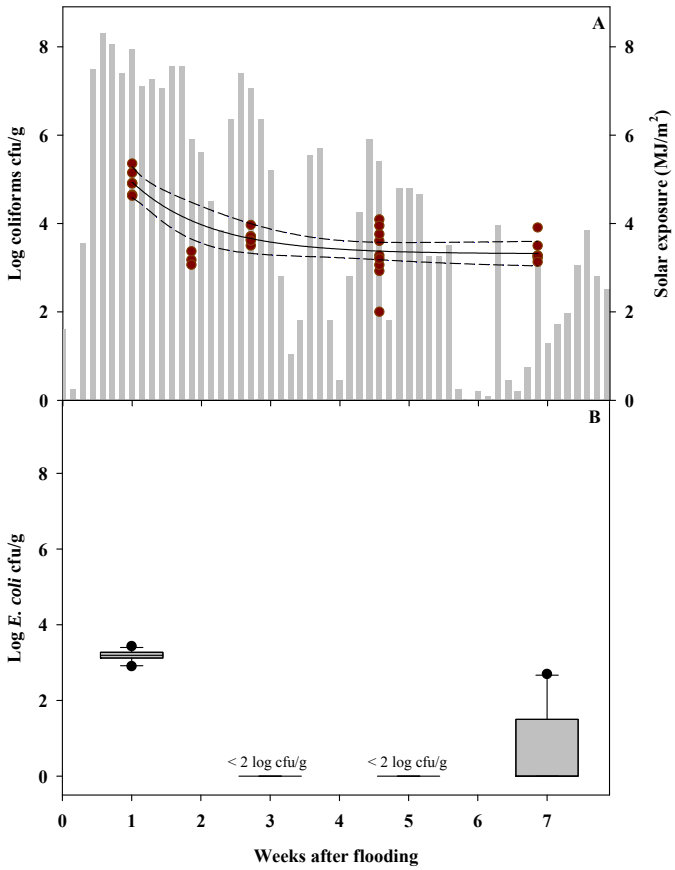

Figure 4.3. (A) Changes in coliforms (log CFU/g) in soil (dots) and solar exposure (bars) after the flooding event. Solid line represents best fitted equation and dot lines are confident bands generated by non-linear regression analysis. **(B)** Boxplot of *E. coli* (log CFU/g) in soil after flooding event. Bottom and top of the box are the 25th and 75th percentile and the end of the whiskers are the minimum and maximum of all the data. Dots represent outlier values.

However, soil samples taken 7 weeks after flooding, were positive for *E. coli*, although in lower levels than those found during the first week of sampling. This specific contamination with *E. coli* did not seem to be related to the flood event and could be due to contamination with faecal material from wild and domestic animals (Gil et al., 2015). Domestic animals such as cattle, sheep, chickens, dogs, cats and horses can contaminate crops with faeces if they pass through growing areas. However, while domestic animals may be separated from growing operations, it can be more difficult to control access by wild animals (e.g. frogs, lizards, snakes, rodents, badgers, foxes, deer

or wild boar) and birds (Harris et al., 2003; Lowell et al., 2010). Lettuce fields sampled within this study were surrounded by several farms, mostly swine farms, which may have contributed to the contamination of the flooded areas. Contamination of agricultural soils after flooding has been previously reported by Casteel et al., (2006), who found high levels of faecal contamination in agricultural soils after an extensive flooding in North Carolina. In this case, a number of samples were positive for the presence of faecal coliforms, *E. coli* and coliphages, indicating the presence of human or animal faeces. Other studies indicated that usually, it takes 2–3 months for enteric bacteria to significantly decrease in soil because of the impact of temperature, solar radiation, and other soil characteristics (Bitton & Gerba, 1984; Manios et al., 2006). In our study, coliforms counts quickly declined in soil samples when compared to available literature. These values were satisfactorily fitted by the nonlinear regression analysis with a R^2 of 0.72 and a S_{yx} of 0.41 (**Table 4.3**). Taking into account the insolation levels (6-8 MJ/m^2) detected after flooding, the microbial decrease can be explained by the climatic conditions (**Figure 4.3**). CDC (2011) reported that microbial survival in soil and the resulting potential for human exposure is difficult to predict because of the variability in the environmental factors related to solar radiation, relative humidity and wind that influenced the humidity of the soil. Under extreme weather conditions, the internalization of *Salmonella* Typhimurium in lettuce has been shown to occur when the soil was inoculated with high levels of bacteria (8-9 log CFU/g soil) (Ge et al., 2012). These authors explained that flooding increased the permeability of the root membranes and caused more nutrients to leak and facilitate the microbial growth.

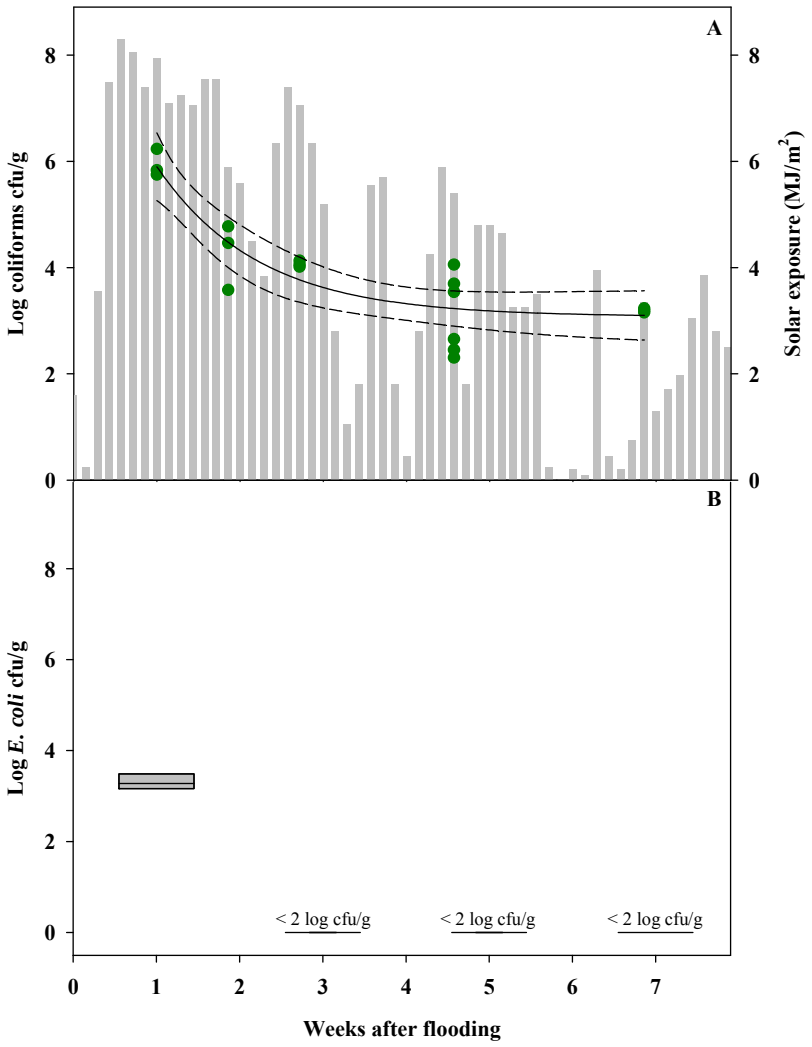

Figure 4.4. (A) Changes in coliforms (log cfu/g) in lettuce (dots) and solar exposure (bars) after the flooding event. Solid line represents best fitted equation and dot lines are confidence bands generated by non-linear regression analysis. **(B)** Boxplot of *E. coli* (log cfu/g) in lettuce after flooding event. Bottom and top of the box are the 25th and 75th percentile.

Figure 4.4 shows coliforms and *E. coli* counts for the lettuce 1, 3, 5 and 7 weeks after flooding. Recently, EFSA (EFSA, 2014a) reported that between 50% and 99.7% of leafy greens sampled in the EU contained less than 10 CFU *E. coli*/g, between 0% and 16% contained more than 10^2 *E. coli* CFU/g, and between 0% and 0.8% contained more than 10^3 CFU *E. coli*/g. Compared to previous published data, higher levels of *E. coli* (> 3 log CFU/g) were found in lettuce samples taken 1 week after flooding. The *E. coli*

concentrations found in lettuce correlated well with those levels observed in irrigation water and soil. Therefore, flood water seemed to be the most feasible vector of contamination with *E. coli* in this study. As previously observed in water and soil samples, coliforms and *E. coli* quickly declined to levels already reported for lettuce (**Figure 4.4**), confirming that survival profiles of faecal microorganisms are usually low in open field (Holvoet et al., 2014a). Microbial decrease in lettuce was satisfactorily fitted by a nonlinear regression analysis with a R^2 of 0.80 and a S_{yx} of 0.52 (**Table 4.2**). In this case, the specific contamination observed in soil samples 7 weeks after flooding was not correlated with the counts in the lettuce.

3.2 Pathogens

Presence of *L. monocytogenes* was not detected in lettuce samples, except for 2 samples collected 3 weeks after the flood event (**Table 4.3**). Leafy greens are known to support the growth of *L. monocytogenes* and many research studies have confirmed this (Beuchat, 1996; Cho et al., 2004; Johannessen, et al., 2002). Crépet et al. (2007) reported that the probabilities of fresh unprocessed and minimally processed vegetables being contaminated with concentrations higher than 1, 2, and 3 log viable *L. monocytogenes* organisms/g were 1.44, 0.63, and 0.17%, respectively. Recently, EFSA (2013) published that prevalence of *L. monocytogenes* in food of non-animal origin (FoNAO) between 2005 and 2011 was 2.7 %. Based on the negative results obtained in this study, the flood water did not seem to represent a source of contamination by *L. monocytogenes*.

One week after the flood event, most of the samples (8 out of 10) of irrigation water, soil and lettuce were positive for *Salmonella* spp. by multiplex PCR (**Table 4.3**). From these samples, only 2 samples of soil and 1 sample of irrigation water were

confirmed by isolation of colonies in culture media. The results obtained highlighted flooding as a potential risk of contamination with *Salmonella* spp. The concentration of foodborne pathogens in flood water has been reported to depend on several factors such as 1) the kind of sources contributing to the contamination, 2) the volume of contaminants released and 3) the degree of their dispersion in the environment (CDC, 2011). As previously mentioned, there were several swine farms in the area affected by flooding, which might contribute to the *Salmonella* spp. contamination. The prevalence of *Salmonella* spp. quickly declined with time in all of the samples. Thus, 3 weeks after the flood event, only 4 out of 18 samples were still positive for *Salmonella* spp. by multiplex PCR. However, this prevalence (22.2 %) was still much higher that the *Salmonella* spp. prevalence reported by EFSA (2013), which in the case of FoNAO was 0.48 % between 2004 and 2011. Gorski et al. (2011) reported a prevalence of *Salmonella* spp. of 2.3 % in the environment around Monterey County in California, which is a major agricultural region of the United States. On the other hand, all the samples tested 5 and 7 weeks after flooding were negative for *Salmonella* spp. Thus, as previously described for *E. coli* spp., the survival profile of *Salmonella* spp. was relatively low, compared with data previously published. In fact, Islam et al. (2004c) reported that *Salmonella* persisted for 23 weeks in soils amended with contaminated composts (inoculated with 7 log CFU/g) and was detected for up to 9 weeks on lettuce. More recently, Kisluk & Yaron (2012) reported that irrigation with contaminated water containing 8.5 log CFU/mL resulted in persistence of S. Typhimurium on parsley for at least 4 weeks. However, irrigation with less contaminated water (2.5 log CFU/mL) resulted in the persistence of the bacteria on the plants for only 48 h. Therefore, depending on the level of contamination and the environmental conditions, mainly high solar radiation, the survival profile of *Salmonella* spp. will probably vary, making it difficult to generalize the consequences and provide recommendations (CDC, 2011).

Positive samples for Verotoxigenic *E. coli* (0145, O111, 0103 and O126) by multiplex PCR were found in 4 soil and lettuce samples out of 10 samples taken 1 week after flooding. However, none of the tested samples was positive for *E. coli* O157:H7. Positive samples were not confirmed by isolation of colonies in culture media. Even though water is usually highlighted as the most probable vector of contamination (Gorski et al., 2011), no positive water samples for VTEC were found in this study. Therefore, the implication of floodwater as the source of VTEC contamination of soil and lettuce was not clear. Though *E. coli* O157:H7 is the most important VTEC serotype in relation to public health, other serotypes have been also involved in sporadic cases and outbreaks (WHO, 2011). In fact, the involvement of non-O157:H7 VTEC strains in human foodborne outbreaks have increased dramatically in the last two decades. Positive samples for non-O157:H7 VTEC strains were only detected in soil 3 and 7 weeks after flooding. However, no positive samples were found 5 weeks after flooding. Therefore, the positive samples identified 7 weeks after the flood event could be associated with the high *E. coli* spp. found in soil samples, which was probably not related to flooding. In general, the results obtained agree with previous studies which suggested the need for mitigation strategies to protect crop-growing areas from incidents capable of releasing faecal material, such as the construction of ditches and establishment of buffer areas (Casteel et al., 2006; EFSA, 2014a). When plants are contaminated in the field with foodborne pathogens such as *E. coli* O157, the populations decline quickly and greatly on healthy lettuce plants, but survive on damaged plants because of the release of nutrients (Aruscavage et al., 2008). Thus, the level of contamination and the time in the growing cycle at which the possible contamination by the flood event took place indicated that the contamination of the plant at harvest was unlikely.

Table 4.3. Microbial results of pathogen detection in water, soil and lettuce samples. Results are expressed as presence or absence in 25 g (soil and lettuce samples) or 100 mL. Results are presented as C: number of positives samples confirmed by conventional methods; P: number of positives samples detected by multiplex PCR; A, number of samples analysed for the presence/absence of pathogens; and na: not analysed.

Weeks after	*L. monocytogenes*			*Salmonella*			*E. coli* O157:H7			*E. coli* O26, O103, O111 & O145		
	Water	Soil	Lettuce	Water	Soil	Lettuce	Water	Soil	Lettuce	Water	Soil	Lettuce
			C/P/A	C/P/A	C/P/A	C/P/A	C/P/A	C/P/A	C/P/A	C/P/A	C/P/A	C/P/A
1	na	na	0/0/2	1/2/4	2/4/4	0/2/2	0/0/4	0/0/4	0/0/2	0/0/4	0/2/4	0/2/2
3	na	na	2/2/6	0/2/6	0/0/6	0/2/6	0/0/6	0/0/6	0/0/6	0/0/6	0/2/6	0/0/6
5	na	na	0/0/6	0/0/4	0/0/6	0/0/4	0/0/4	0/0/6	0/0/4	0/0/4	0/0/4	0/0/6
7	na	na	0/0/6	na	0/0/4	0/0/2	Na	0/0/4	0/0/2	na	0/2/4	0/0/2

3.3 Correlations

Correlations between *E. coli* levels in water and soil with those in lettuce were determined. *E. coli* counts in water were positively correlated with *E. coli* levels in lettuce (R^2=0.998). However, *E. coli* loads in soil had a lower correlation with *E. coli* levels in the lettuce (R^2=0.655). Additionally, correlation between the data obtained for hygiene indicator microorganisms and prevalence of pathogens obtained by multiplex PCR was carried out to determine the predictive value of indicator microorganisms (**Figure 4.5**). A positive correlation was found between the pathogen presence (*Salmonella* spp. and VTEC*)* and the indicator count. This correlation was significant in the case of *E. coli* ($P < 0.005$) and coliforms counts ($P < 0.05$) but it was not significant for *Enterococcus* (**Figure 4.5**).

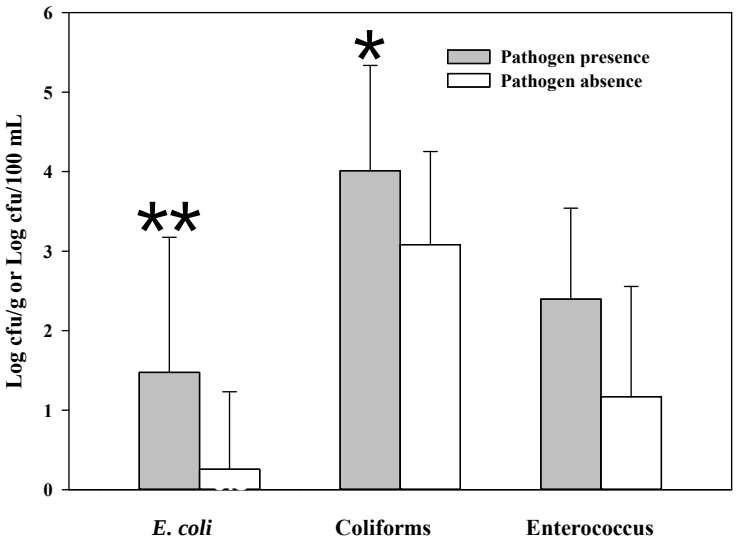

Figure 4.5. Mean values of *E. coli*, Coliforms or *Enterococcus* enumeration (if analysed) in the subset of samples with either absence or presence of pathogens is shown for all the analysed samples (n=70). Pathogen's occurrence included positive signals by GeneDisc® PCR of *Salmonella* and VTEC samples. Significant differences between pathogen absence or presence were determined by Mann-Whitney test represented by * at P-value < 0.05 and ** at P-value < 0.005.

Previous studies did not find a good correlation between loads of microbial indicators of faecal contamination and the presence of foodborne pathogens, although some predictive value has been reported, especially in water, between the faecal indicators and pathogens (Harwood et al., 2005; Schets et al., 2005; Wilkes et al., 2009; Holvoet et al., 2014a). However, based on the results obtained as well as available literature, it could be concluded that the probability of detection of any pathogen is high at high levels of indicators (Savichtcheva & Okabe, 2006; Holvoet et al., 2014a).

4. Conclusions

It could be concluded that flooding affected the microbial quality of lettuce increasing the levels of both indicator and pathogenic microorganisms. After 1 week of the flood event, several samples of water, soil and lettuce were positive for *Salmonella*

spp. by multiplex PCR, evidenced that flooding was an important risk of contamination with *Salmonella* spp. However, very low prevalence of *L. monocytogenes* was observed after flooding, indicating that the floodwater did not seem to represent a source of contamination by this pathogen. Levels of indicator microorganisms and prevalence of pathogens considerable decreased after 3 weeks of the flooding event. The reduction of microorganisms in lettuce and environmental samples was correlated with the high solar irradiation during the days after the flooding. Additionally, a positive correlation was observed between the levels of *E. coli* and the prevalence of foodborne pathogens. Based on the obtained results as well as available literature, it could be concluded that the probability of detection of pathogens was high at high levels of indicators. There was also a correlation between *E. coli* and sample sources, exemplified by the positive correlation between *E. coli* count in water and lettuce. The results obtained in the present study confirm previous knowledge, which defined flooding as a main risk factor for the microbial contamination of leafy greens. However, based on the obtained results the climatological factors during and after the flooding event considerably affect microbial survival in leafy greens.

Chapter V.

Quantitative exposure assessment of *Escherichia coli* in baby spinach primary production in Spain: Effects of weather conditions and agricultural practices.

1. Introduction

Foodborne pathogens such as pathogenic Escherichia coli and Salmonella spp. are presumed to have a low prevalence (<1%) on leafy greens (EFSA, 2013; Holvoet et al., 2014, 2015). Thus, contamination of leafy greens can be considered as a 'rare' event and direct pathogen screening is likely to be ineffective (EFSA, 2014). The prohibitive cost and time consumption of pathogen detection make microbial indicators a good strategy to characterize microbial contamination in the environment of field cultivation and fresh produce (Park et al., 2013). This is the case of *E. coli*, where presence is indicative of conditions favourable for survival of enteric pathogens on fresh produce (Delbeke et al., 2015; Park et al., 2013). *E. coli* has been identified as suitable for a hygiene criterion at primary production of leafy greens and can be applied for validation and verification studies of Good Agricultural Practices (GAP) (EFSA, 2014; Delbeke et al., 2015; Holvoet et al., 2014, 2015).

Quantitative microbial risk assessment (QMRA) using scenario analysis and predictive microbiology constitutes a useful approach in the ongoing efforts to manage food safety risks (Bassett et al., 2012). Several studies have focused on the development of QMRA for enteric pathogens such as *E. coli* O157:H7 and *Salmonella* spp. in leafy greens at field level (Franz et al., 2008a; McKellar et al., 2014), from harvest to retail (Koseki and Isobe, 2005), from farm-to-consumption chain for spinach associated with *E. coli* O157:H7 (Danyluk and Schaffner, 2011), lettuce associated with Listeria monocytogenes (Ding et al., 2013) and in a specific distribution system (Franz et al., 2010; Tromp et al., 2010; Pérez-Rodriguez et al., 2011). A QMRA study was also published related to STEC and Salmonella in leafy greens eaten as salads (Pielaat et al., 2014). However, the low prevalence of foodborne pathogens in leafy greens makes

validation of QMRA with experimental data obtained in commercial set-ups impossible and consequently, the risk associated with consumption of field-grown crops is difficult to assess (McKellar et al., 2014). Up to now, no Quantitative Microbial Exposure Model (QMEM) was developed on the prevalence and levels of *E. coli* on baby spinach at primary production. Franz et al. (2008a) developed a risk model to estimate the probability of lettuce contamination with *E. coli* O157:H7 from manure-amended soil under constant environmental conditions and based on this work the same authors constructed a model to evaluate the degradation of *E. coli* in manure (Franz et al. 2008b, 2010). A QMEM on *E. coli* could represent a good approach to evaluate the impact of different on farm intervention strategies on the distribution of the *E. coli* contamination on leafy greens and correlate this with the potential prevalence of foodborne pathogens.

Leafy greens are grown and harvested all year round under a wide range of agricultural practices and climatic conditions. The microbial ecology on the leaf surface is subjected to constant change mostly due to a daily exposure to environmental and meteorological factors (Vorholt, 2012). The use of a QMEM to assess the impact of changes in weather conditions and agricultural practices on the safety of leafy vegetables might help to design the best preventive measures and intervention strategies to reduce microbial risks. Thus, the objective of this work was to build up a QMEM to evaluate the impact of weather conditions (e.g. seasonality, solar radiation and rainfall), different agricultural practices (e.g. water quality and irrigation system) as well as bacterial fitness in soil on the *E. coli* levels on open-field grown baby spinach at harvest. The situation in the South-East region of Spain, as an example of an intensive agricultural region of fresh produce in Europe, has been taken as a basic condition for this model.

2. Materials & Methods

In this study, the modular process risk model (MPRM) presented by Nauta (2002) and applied e.g. by Daelman et al. (2013) was used as a framework for the QMEM of *E. coli* on leafy greens. **Figure 5.1** illustrates the model, including the different steps in leafy green production such as sowing, growing and harvesting as well as the factors affecting the final concentration of *E. coli* on the harvested baby spinach. The identification of risk factors was based on literature study (e.g. Park et al., 2012, 2013; EFSA, 2014; Gil et al., 2015).

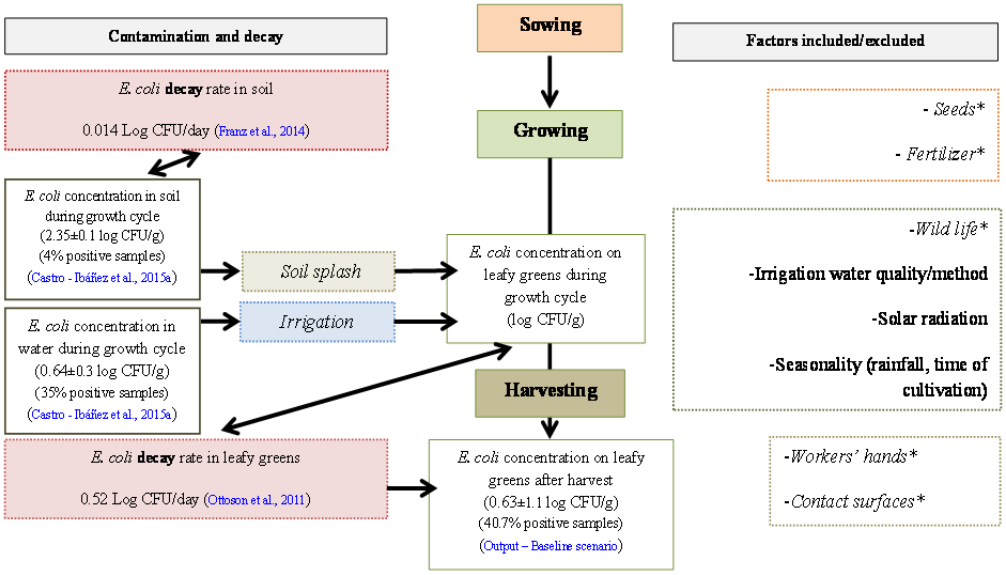

Figure 5.1. Flowchart of the QMEM for the cultivation of leafy greens in open field including decay rates, concentrations, prevalence and considered risk factors influencing the generic *E. coli* concentration on the leafy greens (* excluded risk factors from the model).

However, not all previously described risk factors were taken up in the present model. Focus was made on potential systematic contamination routes avoiding occasional contamination events such as wildlife, contact surfaces of equipment during harvest and workers (**Figure 5.1**).

Contamination attributed to wildlife is random and an unpredictable factor, which makes quantification very difficult (Liu et al., 2013). In relation to cross-contamination by contact surfaces (e.g. hands, harvesting equipment, storage bins), it is bound to occur at this stage of the chain. However, the potential transfer of bacteria of harvest-related events on leafy vegetables has not been examined to date and there is a lack of information on this stage (Park et al., 2013). Additionally, results derived from sampling of contact surfaces (n = 81) and hands (n=27) during harvest in Spain were below the detection limit of 1.7 log CFU/50 cm^2 for *E. coli* (Castro-Ibáñez et al., 2015a).

Previously published research studies have highlighted the potential risks associated to manure used as fertilizer for leafy greens (Islam et al., 2004; Stocker et al., 2015). In this study, the potential risk associated to manure as fertilizer has not been considered as such but it has been included as part of the manure-amended soil because the mix of manure with soil before sowing was reported to be a common practice (Castro-Ibáñez et al., 2015a; Stocker et al., 2015). Seeds have been described as a potential source of pathogenic bacteria (Van der Linden et al., 2013). If the seeds were contaminated, they could theoretically contaminate the plant and the soil. However, there is little information available about the prevalence and pathogen levels of naturally contaminated seeds (Erickson et al., 2014). In this study, contamination coming from seeds was left out also because in a previous study seeds were not identified as a potential source of contamination (Castro-Ibáñez et al., 2015a). Therefore, taking into account that not all potential risk factors were included in this model, the outcome of the proposed QMEM will not be able to predict the full actual situation of the *E. coli* contamination on leafy greens but it might give valuable information regarding the current situation in this intensive agricultural region of Europe. To build the model, data

were obtained from different sources including scientific literature, experimental data, expert opinion and company information. Data for *E. coli* concentration of irrigation water and manure-amended soil were taken from previous publications based on systematic sampling studies (Holvoet et al., 2014; Castro-Ibáñez et al., 2015a,b). Where needed, additional experimental data were collected and further described in the model building. Company information was collected from the three major Spanish producers of fresh produce. Expert opinion by internal discussions within a research project consortium of the FP7 project Veg-i-Trade (www.vegitrade.org) were if needed, included.

2.1 Model software and simulations

The model was constructed in Excel and implemented in @Risk software (V 6.3.1. Palisade Corporation, US). Best fitting distribution @Risk software was applied and selection was based on Chi Square statistics and P-P plots. Each simulation consisted of 100,000 Monte Carlo iterations using Latin Hyper cube sampling. Stability of the model and number of iterations were tested by running the baseline scenario three times.

2.2 Baseline scenario

The overview of the baseline QMEM was summarized in **Table 5.1** where inputs with a brief description, applied value or distribution, units and references are shown. The baseline model includes cultivation of baby spinach in open fields during the spring period (March – April), assuming a regular growing period, solar radiation (intensity and duration) and rainfall for this time of the year.

Table 5.1. Overview of inputs and distributions for QMEM of *E. coli* on leafy greens (baseline scenario, i.e. spring as cultivation period, sprinkler as irrigation method and microbial water quality based on three mayor Spanish producers).

Cell	Variable	Abbreviation	Value	Unit	Source
C1	**Distribution randbetween (0,1)**	Randbetween	0 - 1	-	Calculated
C2	**Fraction above detection limit (3/81)**	SoilD	0.04	-	Castro-Ibáñez et al. 2015a
C3	***E. coli* concentration soil**	SoilConc	IF(C1>C2,0.7,2.35)	log CFU/g	Calculated
C4	**Growing time in spring**	Days_growth	RiskPert(32,36.8,43)	Days	Company info
C5	**Soil transferred by irrigation**	Transfer_soil_splash	RiskBetaGeneral (0.5,1.2,0.06,16.7)	gr soil/gr produce	In house experiment
C6	**Bacteria transferred from soil to plant**	Bacteria_transfer	RiskUniform(0.35,0.9)	%	Girardin et al. 2005
C7	**Probability of splashing**	Splash_prob	RiskPert(0.02,0.04,0.06)	%	In house experiment
C8	***E. coli* concentration on plant after splashing by irrigation**	Ecoli_irrigation_splash	C3 * C9 * C5 * C6 * C7	log CFU/g	In house experiment
C9	**Number of irrigation moments**	Irrigation_moments	C4 - C9	Days	Calculated, Company info
C10	**Rain days during growth**	Number_rain_days	RiskPert(1,5.6,14)	Days	Calculated
C11	***E. coli* concentration on plant after splashing by rain**	Ecoli_rain_splash	C3 * C10 * C5 * C6	log CFU/g	Calculated
C12	***E. coli* concentration on plant after splashing (irrigation + rain)**	Ecoli_splashing_irrigation_rain	C8 + C11	log CFU/g	Calculated
C13	**Distribution randbetween (0,1)**	Randbetween	0 - 1	-	Calculated
C14	**Fraction above detection limit (27/78)**	WaterD	0.35	-	Calculated
C15	**Distribution positives**	WaterConc	RiskExtvalue(0.5,0.2)	-	Castro-Ibáñez et al. 2015a
C16	***E. coli* concentration in irrigation water**	Ecoli_conc_irrigation	IF(C13>C14,0,C15)	log CFU/100ml	Calculated
C17	**Amount of water transferred to the plant during irrigation**	Transfer_water	RiskUniform(1.8, 21.6)	ml/g	In house experiment
C18	***E. coli* concentration on plant after irrigation**	Ecoli_after_irrigation	C17*C18*C9/100	log CFU/g	Calculated
C19	***E. coli* concentration on plant after splashing and irrigation**	Ecoli_irrigation_splashing	C12 + C18	-	-
C20	**Hours of sun spring**	Sun_hours	RiskPert(5,10.4,12)	-	Calculated
C21	***E. coli* concentration on plant at harvest**	Ecoli_harvest	C19-0.52*C4*(C20/24)	log CFU/g	Ottoson et al. 2011
C22	**IF function to truncate the data**	Ecoli_harvest_truncate	IF(C21>4,4,IF(C21<0,0,C21))	log CFU/g	Calculated

The spring cultivation period (number of days between sowing and harvest) was provided by the company and ranging between 32 and 49 days (*Days_growth*, **Table 5.1**) while winter season ranging between 50 and 66 days (*Days_growth*, **Table 5.2**). The average days of rain for each season were obtained from the local climatological database of that area for the years 2005 till 2015 (SIAM, 2015). In general, climatic data (e.g. solar radiation and rainfall) were fitted to a Pert distribution (min, most likely, max), mostly due to the large amount of available data (SIAM, 2015).

In the baseline model, contamination coming from manure-amended soil and irrigation water were considered to be stable during the whole cultivation period because no differences for *E. coli* concentration or prevalence during the cultivation were noticed in previous studies (Castro-Ibáñez et al., 2015a). Average levels of *E. coli* in the irrigation water currently applied in the South-East region of Spain, were included as an input of this model (**Figure 5.1**). As irrigation system, sprinkler irrigation (overhead) was selected for the baseline scenario of baby spinach production.

2.3 Risk Factors

2.3.1 Soil

The distribution of the *E. coli* levels in the manure-amended soil (*SoilConc*, **Table 5.1**) was based on 81 samples taken during cultivation in the South east region of Spain (Castro-Ibáñez et al., 2015a). Therefore, a Randbetween function between 0 and 1 (or 0 and 100% of the samples) was introduced based on the above mentioned data (**Table 5.1**). In this approach, a random number between 0 and 1, was generated for each iteration in the simulation. As a certain fraction (3/81) was above the detection limit for *E. coli* (2.0 log CFU/g), the IF function was applied to construct the distribution. If the obtained random number was below the reported positive fraction

(4%), the estimated *E. coli* concentration corresponded to the previously published average level for manure-amended soil samples (Castro-Ibáñez et al., 2015a). If the obtained random number was above the reported positive fraction (4%), the estimated *E. coli* concentration was consider to be under the detection limit. However, the detection limit described in the study previously performed in this region of Spain (Castro-Ibáñez et al., 2015a) was unusually high (2.0 log CFU/g), while other research studies reported lower detection limits (0.7 log CFU/g) (Holvoet et al., 2014). Therefore, in order to have a more realistic value of the *E. coli* levels in the negative samples of manure-amended soil, a combination of the two previously published detection limits was taken into account. To do so, a distribution (RiskUniform, **Table 5.1**) was introduced to generate a concentration between 0.7 log CFU/g (Holvoet et al., 2014) and 2.0 log CFU/g (Castro-Ibáñez et al., 2015a) for the negative samples. The *E. coli* levels of both fractions of the manure-amended soil (below or above the positive fraction) were included in the model as initial manure-amended soil contamination. In the current QMEM, it was assumed that produce contamination from manure-amended soil was mostly caused by soil splashing during irrigation (sprinkler irrigation) and rainfall. In order to estimate the probability of soil splashing per gram of produce as well as the amount of manure-amended soil transferred during each irrigation event, field experiments were conducted. To obtain the data, five sampling areas located at 0 cm, 50 cm, 100 cm, 200 cm and 400 cm from the irrigation sprinklers of a real-case baby spinach field were selected and irrigated for 20 min. For the estimation of the splashing probability and transfer of manure-amended soil during irrigation, a total of 1,500 leaves were randomly taken between the five selected zones. Presence and absence of manure-amended soil on the surface was determined. A RiskPert distribution was used to determine the probability of splashing (*Splash_prob,* **Table 5.1**). To

evaluate the amount of soil transferred to the leaf surface, 50 leaf samples were randomly taken before and after the irrigation event in each of the 5 selected sampling zones). The leaves were collected and the irrigation water was let air dry. Once dried, the amount of manure-amended soil remaining on the leaf surface as well as the leaf weight after removing the dried soil were determined using a precision scale (ENTRIS 3202-1S, Sartorius, Germany). Outliers of the obtained data were removed using SPSS Statistics (IBM) and based on 72 data points; a Betageneral distribution (*Transfer_soil_splash*, **Table 5.1**) was made (**Figure 5.2A**).

Regarding the probability of rain splashing, it was assumed to be 100%, indicating that there was always manure-amended soil splashing during rainfall. Data related to quantitative transfer of bacteria from manure-amended soil to plant tissue due to soil splashing was taken from Girardin et al. (2005), who quantitatively assessed the transfer of two pathogen surrogates on parsley. Adaptations from parsley to baby spinach leaves were made mostly due to differences in the surface area and weight between parsley and baby spinach leaves. With that purpose, the surface of the baby spinach leaves were measured using Image J (NIH image, National Institute of Health, US) and weighted using a precision scale to build up a distribution for the % of bacteria transferred from manure-amended soil to leaves (log CFU/g) (*Bacteria_transfer*, **Table 5.1**). Based on that, the distribution of *E. coli* concentration on baby spinach due to soil splashing during an irrigation/rain event was calculated (*Ecoli_irrigation_splash*, **Table 5.1**).

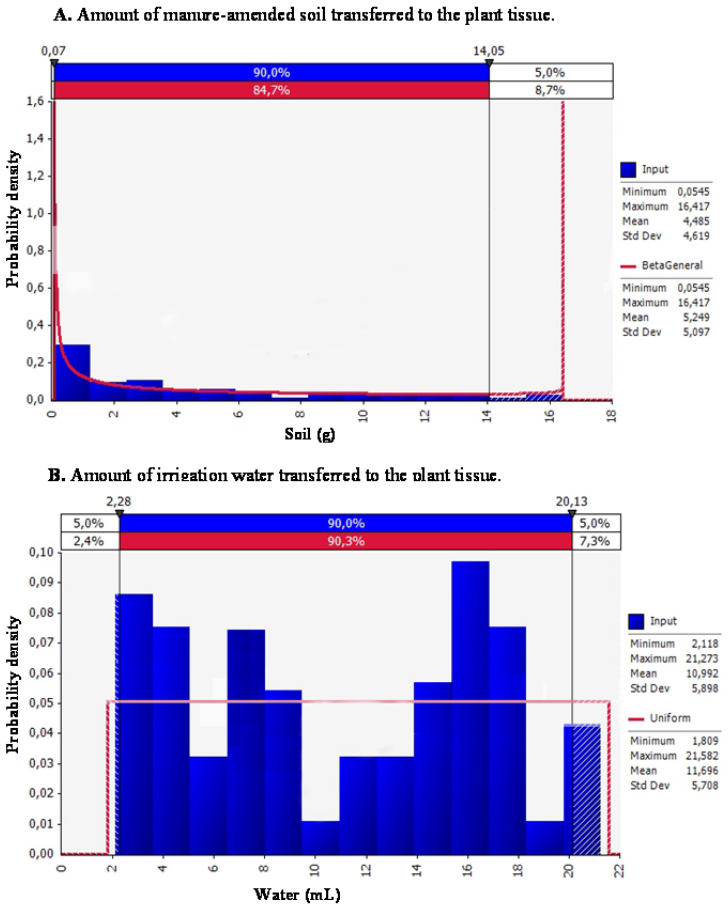

Figure 5.2. Input data distribution for the water volume transferred to the plant by overhead irrigation **(A)**. Input data distribution for the amount of soil transferred to the plant by splashing during irrigation **(B).**

2.3.2 Irrigation Water

The distribution of the *E. coli* levels in irrigation water (*WaterConc*, **Table 5.1**) was based on 78 samples taken during cultivation in the South east region of Spain (Castro-Ibáñez et al., 2015a). Therefore, a Randbetween function between 0 and 1 (or 0 and 100% of the samples) was introduced based on the above mentioned data (*WaterD*, **Table 5.1**). As a certain fraction (27/78) was above the detection limit for *E. coli* (0 log CFU/g), the IF function was applied to construct the distribution. If the obtained random number was below the reported positive fraction (35%), *E. coli* levels of irrigation water were determined using previously published data (Castro-Ibáñez et al., 2015a) fitted to a

RiskExtvalue function (*WaterConc*, **Table 5.1**). In order to estimate the amount of irrigation water transferred during each irrigation event, field experiments were conducted. Following the same experimental set-up as previously described for manure-amended soil, five sampling areas located at 0 cm, 50 cm, 100 cm, 200 cm and 400 cm from the irrigation sprinklers of a real-case baby spinach field were selected and irrigated for 20 min. To evaluate the amount of irrigation water transferred to the leaf surface, 75 leaf samples were randomly taken before and after the irrigation event in each of the 5 selected sampling zones. A micropipette (Eppendorf, Germany) was used to collect and weight the water remaining on the baby spinach leaves after irrigation using a precision scale (ENTRIS 3202-1S). The obtained data was fit to a RiskUniform distribution (**Figure 2B**) to determine the amount of irrigation water transferred (*Transfer_water*, **Table 5.1**). Based on company information, a daily irrigation event was included in the model except for the rainy days (*Irrigation_moments*, **Table 5.1**).

2.4 Bacterial fitness

Based on literature, *E. coli* is expected to decay when present on the surface of intact leafy greens during open field production (Solomon et al., 2003; Islam et al., 2004; Stine et al., 2005). However, there is still a lack of experimental data on the inactivation rate as a function of physical factors such as temperature, light intensity and solar radiation. In fact, the decay rate (expressed in log CFU/g/day) reported by Ottoson et al. (2011) was not applicable to our situation due to the constant illumination applied in the climate chamber during their experiments. To solve this problem, original data for the climate chamber experiments were provided by contacting the authors (Ottoson et al., 2011) and the number of hours exposed to sun light was adapted using real climatological data. Based on that, *E. coli* decay on baby spinach (*Ecoli_harvest,* **Table**

5.1) due to solar radiation (intensity, (W/m^2) and duration (t)) in spring followed a first-order kinetic equation with one parameter (*k*): $Log\ N/N_0=0.52$*t, where *t* represented time (days), *0.52* was the daily decay (log CFU/g/day) and N/N_0 represented the bacterial concentration at a certain time (*t*).

Enteric indicator and pathogenic bacteria have been shown to survive for long periods of time in soil (Himathongkham et al., 1999; Jiang et al., 2002). Several authors have also described the bacterial decline in soil, mostly because soil is a secondary habitat for these microorganisms (Durso et al., 2004; Lang et al., 2007; Franz et al., 2014). The impact of *E. coli* decline in the soil on the *E. coli* levels of baby spinach at harvest was determined based on the recently published work of Franz et al., (2014) (**Figure 5.1**).

2.5 Assumptions

In this study, and mostly due to knowledge gaps, some assumptions were needed to allow the completion of a quantitative model. The considered assumptions were based on available literature and company information obtained from the three major Spanish producers of fresh produce. For instance, an assumption was made regarding the probability of splashing of manure-amended soil in the leaves during rain, which was supposed to be 100 %. It was assumed that the environmental temperature did not affect bacterial decay on the plant tissue. Only solar radiation (intensity and duration) was considered to cause bacterial decay in baby spinach based on Ottoson et al. (2011). A fourth assumption was established to exclude unrealistic simulations by truncating the concentration data with a minimum value of 0.0 log CFU/g and a maximum of 4.0 log CFU/g. The minimum and the maximum *E. coli* levels were established based on

previous experimental data assuming regular and extreme climatic conditions (e.g. flooding) (Castro-Ibáñez et al., 2015a).

2.6 Scenarios

Seven different scenarios were considered allowing the study of the impact of weather conditions, agricultural practices and bacterial fitness in soil. Therefore, some modifications in distributions included in the baseline scenario of **Table 5.1** were made and the outcome of each scenario was compared to the baseline scenario previously described in section 2.2. **Table 5.2** summarizes all the changes made for the different scenario simulations. Four scenarios evaluated the impact of different weather conditions including: (Scenario 1) winter season characterized by longer cultivation time, higher rainfall and lower solar radiation; (Scenario 2) no solar radiation, characterized by equal conditions than the baseline scenario without the solar radiation; (Scenario 3) no rain, characterized by equal conditions than the baseline scenario without rainfall; (Scenario 4) flooding, characterized by spring conditions with no irrigation, heavy rainfall and two waiting periods between the flooding event and harvest. A second set of scenarios were related to the impact of agricultural practices: (Scenario 5a, b and c) irrigation water, characterized by equal conditions than the baseline scenario combined with different microbial quality of the irrigation water, i.e. improving water quality by water treatment (treated irrigation water, non-treated irrigation water and potable water) and (Scenario 6) irrigation system, characterized by equal conditions than the baseline scenario but using drip irrigation. Finally, Scenario 7 was built up in order to evaluate the impact of *E. coli* decay in the soil on the final outcome.

3. Results and Discussion

3.1 Impact of weather conditions

3.1.1 Seasonality

Several studies have evaluated the impact of weather and seasonal changes in the microbial loads on fresh produce, but the main driving forces for these variations still remain confusing (Ward et al., 2015). However, it is clear that meteorological conditions have an important impact on the *E. coli* levels on leafy greens at harvest (Park et al., 2015). In spring, characterized by a shorter growth period (5-7 weeks), higher ambient temperatures (15.6±4.1°C) and high solar radiation (184.4±72.0 W/m^2), *E. coli* prevalence and mean levels were higher than in winter, with a longer cultivation period (7-9 weeks), colder temperatures (10.0±2.3°C) and less solar radiation (128.2±38.5 W/m^2) (**Table 5.3**). These differences could be due to a combination of weather conditions such as low temperature and less rain in winter when compared to spring but also due to a longer growing period which might increase the bacterial decay during cultivation. Even though some studies have stated that colder temperatures enhance microbial survival (Oliveira et al., 2012), the longer cultivation periods, the reduced number of irrigation days and the decreased levels of solar radiation during winter might influence *E. coli* levels. Our findings are in agreement with previous studies showing that the highest levels of *E. coli* and the highest prevalence of pathogens in water and produce were observed in that time of the year when the ambient temperature and irrigation water temperature were the highest (e.g. spring in the present case study) (Isobe et al., 2004; Shehane et al., 2005; Strawn et al., 2013; Castro-Ibáñez et al., 2015a; Park et al., 2015). Furthermore, the positive association between temperature and foodborne illness has been demonstrated (Kim et al., 2015).

Table 5.2. Overview of the adaptations and changes made in the baseline scenario for the different scenarios evaluating impact of weather conditions and agricultural practices.

Scenario	Variable	Abbreviation	Value	Unit	Source
		Weather conditions			
Winter (Scenario 1)					
C4	**Growing time in winter**	**Days_growth**	RiskPert(51,56.7,67)	Days	Calculated, Company info
C9	**Number of irrigation moments**	**Irrigation_moments**	C4 - C9	Days	Calculated, Company info
C10	**Rain days during growth**	**Number_rain_days**	RiskPert(3,10.6,23)	Days	Calculated
C20	**Hours of sun winter**	**Sun_hours**	RiskPert(6,8.5,11)	-	Calculated
C21	***E. coli* concentration at harvest**	**Ecoli_harvest**	C19-0.48*C4*(C20/24)	log CFU/g	Ottoson et al. 2011
No solar radiation (Scenario 2)					
C21	***E. coli* concentration at harvest**	**Ecoli_harvest**	C19-0.18*C4	log CFU/g	Ottoson et al. 2011
No rain (Scenario 3)					
C9	**Number of irrigation moments**	**Irrigation_moments**	C4	Days	Calculated, Company info
C10	**Rain days during growth**	**Number_rain_days**	0	Days	Calculated
Flooding event (Scenario 4)					
C10	**Time between flooding and water evaporation**	**Time_flooded_spring**	RiskPert(1,3,10)	Days	Castro-Ibáñez et al. 2015b, Company info
C21	***E. coli* concentration after 7 days**	**Ecoli_after7days**	C19-0.52***7***(C20/24)	log CFU/g	Calculated, Ottoson et al. 2011
C21	***E. coli* concentration after 21 days**	**Ecoli_after7days**	C19-0.52***21***(C20/24)	log CFU/g	Calculated, Ottoson et al. 2011
		Agricultural practices			
Treated water (Scenario 5a)				-	
C14	Fraction above detection limit	**WaterD**	0.11	-	Castro-Ibáñez et al. 2015a
C15	Distribution positives	**WaterConc**	RiskExtvalue(0.44,0,12)	-	Castro-Ibáñez et al. 2015a
Non treated (Scenario 5b)				-	
C14	Fraction above detection limit	**WaterD**	1	-	Castro-Ibáñez et al. 2015a
C15	Distribution positives	**WaterConc**	RiskLogistic(0.65,0.2)	-	Castro-Ibáñez et al. 2015a
Potable water (Scenario 5c)					
C15	Distribution positives	**WaterConc**	0	-	(EC) 852/2004
Drip irrigation (Scenario 6)					
C9	Number of irrigation moments	**Irrigation_moments**	0	Days	Company info

Table 5.3. Overview of weather conditions influencing the *E. coli* distribution on leafy greens at harvest (log CFU/g) and fraction of negative samples (%) in different simulated scenarios.

Scenario	Mean	Stdv	P50	P75	P90	P99	P99.9	% negatives
Seasonality (spring versus winter) – Scenario 1								
Spring (baseline scenario)	0.63	1.11	0.00	0.88	2.48	3.30	4.00	59.30
Winter	0.48	1.00	0.00	1.32	2.10	3.00	4.00	69.20
Solar radiation – Scenario 2								
Spring (no solar radiation)	1.18	1.32	0.86	1.89	3.54	4.00	4.00	41.02
Winter (no solar radiation)	0.40	0.85	0.00	0.00	1.95	3.24	3.58	75.73
Rainfall – Scenario 3								
Spring (no rainfall)	0.03	0.17	0.00	0.00	0.00	1.04	2.02	96.26
Winter (no rainfall)	0.01	0.07	0.00	0.00	0.00	0.38	0.93	97.92
Soil decay – Scenario 7								
Spring (soil decay)	0.47	0.95	0.00	0.46	1.90	4.00	4.00	70.40
Winter (soil decay)	0.50	1.00	0.00	0.37	2.10	3.95	4.00	70.70

3.1.2 Solar radiation

Simulated scenario based on spring conditions but excluding solar radiation showed the important impact that this factor had on the *E. coli* levels at harvest. In the case of spring, the number of positive samples substantially increased when solar radiation was excluded (**Table 5.3**). However, when the winter scenario was evaluated, the exclusion of the solar radiation did not significantly affect the percentage of positive samples (**Table 5.3**). It has been reported that factors such as light intensity, humidity, radiation and temperature have an important influence on *E. coli* survival (Oliviera et al., 2012; Park et al., 2014, 2015). Faster inactivation rates of enteric bacteria have been reported in grass surfaces under both sun and shade conditions during the summer months when compared to winter conditions (Sidhu et al., 2008). Additionally, *E. coli* counts have been reported to decrease exponentially with day length and exposure to insolation indicating that solar inactivation is a critical mechanism for bacterial reduction in the field (Whitman et al., 2004). Similarly, Sinton et al. (2002, 2007)

observed significantly higher inactivation rates of *E. coli* associated with solar radiation in water during summer as compared to winter. It was suggested that these differences were due to variations in temperature and solar indexes between the two seasons.

3.1.3 Rainfall

Simulated scenarios showed that rainfall had a substantial impact on *E. coli* distribution at harvest. In those scenarios where rainfall was not considered (spring no rainfall and winter no rainfall), the mean *E. coli* values and the percentage of positive samples at harvest were significantly decreased when compared to the baseline scenario (**Table 5.3**). In general, water input, either as natural rainfall or irrigation, is a potential vector for bacterial transfer from the manure-amended soil to the plant tissue (Monaghan and Hutchison, 2012). Several studies proved that water splashing onto the fields, can transfer contaminated soil and thus transfer microbial organisms onto the crops (Girardin et al. 2005; Keraita et al., 2007; Cevallos-Cevallos et al., 2012; Monaghan and Hutchison, 2012).

3.1.4 Flooding

Flooding has been described as a potential risk for microbial contamination of fresh produce, especially when the agricultural field is adjacent to livestock farms and industrial and residential areas (Miraglia et al., 2009). FDA (2011) established codes of practice including intervention strategies to be implemented in case of a flood event. In this study, the simulation of a flooding event was evaluated (**Figure 5.3**). Four scenarios were considered after the flooding event (**Table 5.2**): spring and winter conditions with two waiting periods from the flooding event to harvest (7 and 21 days). When a waiting period of 7 days was considered, no significant differences were observed between the spring and winter scenarios regarding the *E. coli* levels with 2.0±0.7 log CFU/g and 2.4±0.7 log CFU/g, respectively, with a higher percentage of positive samples for both

cases (98.9% for spring and 99.9 % for winter). However, when a waiting period of 21 days was considered between the flooding event to harvest, significant differences were observed between the two scenarios (spring and winter). E. coli levels at harvest were 0.0±0.1 log CFU/g and 0.4±0.4 log CFU/g for spring and winter, respectively. Significant differences were also observed for the percentage of positives samples between spring and winter (10.5% and 68%, respectively), confirming previous reports showing the impact of the solar radiation during spring in the bacterial decay (Whitman et al., 2004). These results were in agreement with previous findings on a real flooding event in this region which reported unusual high levels of *E. coli* on lettuce one week after a flooding event that drastically declined after 14 and 21 days (Castro-Ibáñez et al., 2015b).

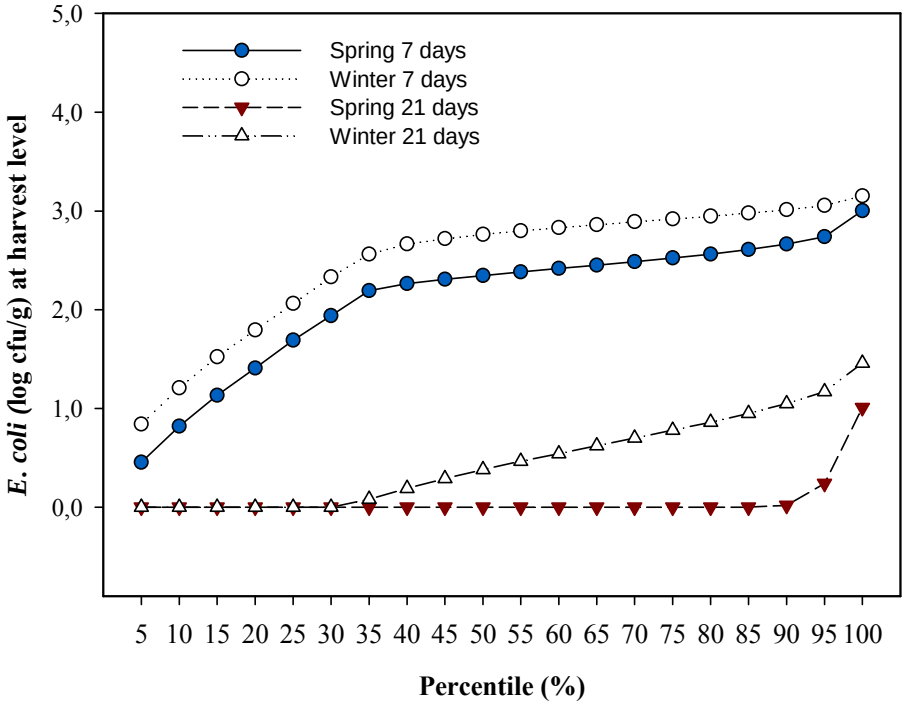

Figure 5.3. Impact of season (spring vs. winter) and holding time (7 vs. 21 days) on the *E. coli* distribution (log CFU/g) in leafy greens at harvest after flooding event.

The differences gained relevance if a longer holding time (21 days) was applied. In this case, the mean values were reduced to 0.03±0.10 log CFU/g and 0.44±0.40 log CFU/g for spring and winter, respectively. Percentage of negatives samples was 89.52% and 31.86% for spring and winter, respectively. These results were in agreement with previous findings on real-event flooding in this region which reported high levels of *E. coli* on the produce one week after the flooding event that drastically declined due to the high solar radiation after the flooding (Castro-Ibáñez et al., 2015b). Furthermore, season had an impact in case of longer holding time during the spring season with higher solar radiation and more sun hours, which caused a faster bacterial decay (Whitman et al., 2004).

3.2 Impact of agricultural practices

3.2.1 Water quality

Water has been identified as a major reservoir for foodborne pathogens and irrigation water has been described as a potential vehicle for transmission of pathogens to environment and fresh produce (Steele & Odumeru, 2004; Söderström et al., 2008; Olaimat & Holley, 2012; Benjamin et al., 2013; Park et al., 2013; Allende & Monaghan, 2015). Human pathogenic microorganism has been shown to survive for several months in river and storage water reservoirs (Manning, 2008; Jawahar and Ringler, 2009). Laboratory and field studies have shown that pathogens and indicator organisms (e.g., *E. coli* O157:H7 and generic *E. coli*) transmitted from irrigation water to produce can remain viable for variable periods of time depending on environmental conditions (Delaquis et al., 2007). Four scenarios have been studied to evaluate the potential impact of irrigation water quality on *E. coli* levels in baby spinach at harvest (**Figure 5.4**). When non-treated surface water was applied as irrigation water, prevalence

(50.7%) and mean *E. coli* levels (1.0±0.8 log CFU/g) were higher than the baseline scenario and the scenario where potable water was used as irrigation water (26.9% and 0.2±0.54 log CFU/g, respectively). The use of surface water treated with potassium permanganate also reduced the prevalence (32.2%) and the mean *E. coli* levels (0.6±0.9 log CFU/g) of baby spinach at harvest. The obtained results confirmed the relevance of irrigation water quality to avoid microbial contamination of leafy greens (Suslow, 2010). The use of water treatments has been previously proposed as a good intervention strategy to reduce the risk of produce contamination with foodborne pathogens and to maintain a constant irrigation water quality all year around (Pachepsky et al., 2011; Gil et al., 2015). In this study, the use of potable water seemed to be a good option to reduce potential contamination of fresh produce via irrigation water as specified in many GAP guidelines (CAC/RCP 1-1969, 2003; CAC/RCP 53, 3003). However, the use of potable water for irrigation is, in most cases, not viable and cost-prohibitive for many farmers, and water treatment is the only option to reduce microbial risks associated to irrigation water (Allende & Monaghan, 2015).

3.2.2 Irrigation method

The likelihood of the edible parts of the plants becoming contaminated during irrigation depends upon a number of factors, including the location of the edible part of the plant on the growing field (e.g., distance from the soil or water surface), the frequency of irrigation, the surface of the edible portion (i.e., smooth, rough, or webbed) and the type of irrigation method (i.e., furrow or flood irrigation, sprinkler, or drip) (Gerba, 2009; Uyttendaele et al., 2015). In this study, results of the QMEM confirmed that sprinkler irrigation showed a higher risk of *E. coli* contamination when compared to drip irrigation (**Figure 5.4**).

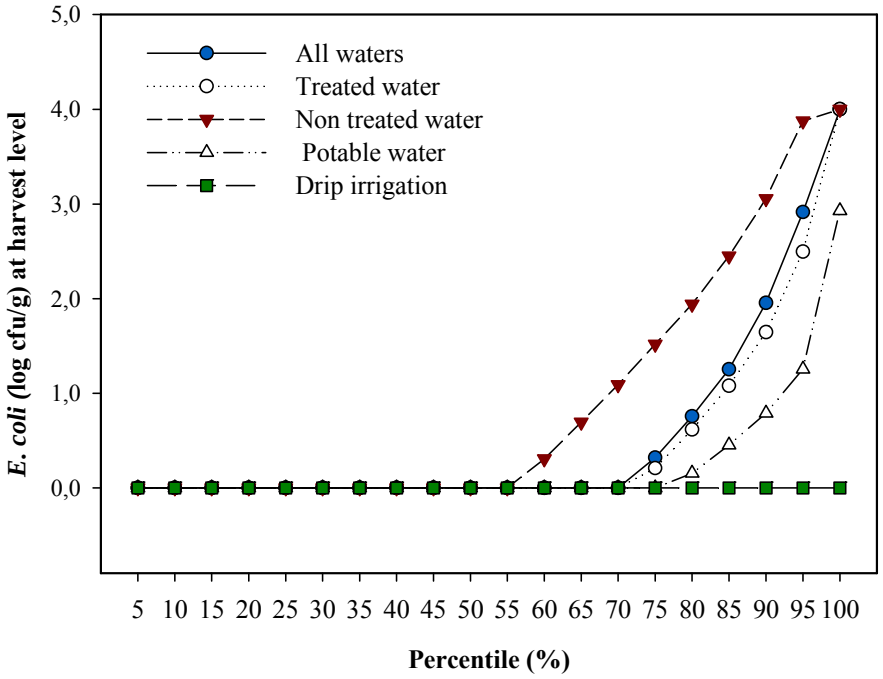

Figure 5.4. Impact of irrigation water quality and irrigation method on the *E. coli* distribution (log CFU/g) in leafy greens at harvest.

In fact, *E. coli* prevalence was decreased from 50.7% to 0% when non-treated surface water was applied using drip irrigation instead of sprinkler irrigation. These results were in agreement with previous research which reported that sprinkler irrigation represented a higher microbial risk mostly due to the direct contact between the irrigation water and the edible part of the plant (Oron et al., 1992; Song et al., 2006).

3.2.3 *E. coli* decay in soil

To evaluate the impact of *E. coli* survival and decline in the soil in the *E. coli* levels of baby spinach at harvest, an additional scenario (Scenario 7) was developed based on the recently published work of Franz et al., (2014). The obtained results (**Table 5.3**) showed that when *E. coli* decline in soil is taken into account, prevalence and mean E. *coli* levels in baby spinach were reduced when compared to the baseline scenario. The obtained results were in agreement with previous studies which showed

that *E. coli* decline in soil seems to be correlated with a decrease in *E. coli* levels on fresh produce (Castro-Ibáñez et al., 2015b).

4. Conclusion

The developed QMEM for *E. coli* concentration on baby spinach at harvest, showed how different factors affect the *E. coli* loads in different scenarios, including weather conditions (e.g. seasonality, solar radiation and rainfall), different agricultural practices (water quality and irrigation system) as well as the impact of the bacterial fitness (e.g. bacterial decay in soil). Based on the obtained results it has been demonstrated that the growing period (season) and consequently, the length of the growing cycle had an important impact on the estimated *E. coli* numbers at harvest.

Among weather factors, solar radiation (intensity and duration) considerably decreased *E. coli* prevalence in baby spinach at harvest. Extreme rainfall, represented by a flooding, significantly increased prevalence and *E. coli* levels on baby spinach. However, the waiting period between the flooding event and harvest seemed to have an important impact decreasing both prevalence and *E. coli* levels. Regarding agricultural practices, the quality of the irrigation water and the irrigation system significantly affected the contamination of baby spinach with *E. coli*. The use of potable water or treated surface water was shown to be the most adequate irrigation waters for leafy greens. Additionally, the use of drip irrigation significantly decreased *E. coli* prevalence and levels even when non-treated surface water was applied. The developed QMEM allowed the assessment of different weather conditions and agricultural practices on the final *E. coli* levels on baby spinach at harvest.

This QMEM might represent a good tool for growers to decide the impact of specific preventive and intervention strategies aiming to reduce microbial contamination. As stated in previous research studies, *E. coli* may be used as a surrogate organism for foodborne bacterial pathogens. Therefore, the results obtained using this QMEM for *E. coli* levels in baby spinach may be used as a proxy or estimate for the potential behaviour of these pathogens under specific conditions.

Chapter VI.

Identification of sampling points suitable for the detection of microbial contamination in fresh-cut processing lines.

1. Introduction

The Centres for Disease Control and Prevention from the United States (CDC) as well as the European Food Safety Authority (EFSA) have reported an increase in foodborne illnesses associated with produce in recent years (CDC, 2010b; EFSA, 2013). The last numbers provided by EFSA indicated that in Europe, outbreaks associated with food of non-animal origin corresponded to 5% of the total foodborne outbreaks in 2007, but this percentage increased to 30% in 2011 (EFSA, 2013). A link between some bacterial pathogens and leafy greens, mostly ready-to-eat (RTE) lettuce and spinach salads, has been shown (Taban & Halkman, 2011). Increased consumption as well as larger scale production and distribution of produce over the past two decades are factors that might have contributed to the increase in the number of foodborne outbreaks (Olaimat & Holley, 2012). The role of these factors in the increment of foodborne occurrences resides, on the one hand, in the possibility of contamination during storage and distribution (Gil et al., 2015) and, on the other hand, in the fact that globalization and large scale production of fresh produce implies longer distribution times and greater distribution distances which entails more complexities (Kirezieva et al., 2015).

Due to the absence of an inactivation step before consumption, products labelled as assumed RTE are potential sources of human pathogens. Available literature highlights that the hygienic status of the raw materials and the processing environment are significant factors for the microbiological safety of RTE products (FDA/CFSAN, 2008; EFSA, 2014a). Contact surfaces and washing steps are potential sources of microbial contamination (Allende et al 2004; Lehto, et al 2011; Holvoet et al., 2012; Zilelidou, et al., 2015). The equipment used for cutting and shredding leafy greens in an industrial processing plant was identified as the source of contamination in an outbreak

investigation (Stafford et al., 2002). A study performed in a pilot scale processing plant demonstrated that one contaminated batch of leafy greens can easily contaminate subsequent batches of previously uncontaminated product if an effective antimicrobial intervention strategy is not implemented (Buchholz et al., 2012a). Intervention measures during processing such as produce sanitation washing; water disinfection and cleaning of food preparation surfaces must be implemented to minimize cross-contamination with pathogenic microorganisms (Gil et al., 2015). Nevertheless, the washing/sanitizing step does not eliminate pathogens effectively when present in the produce and cross-contamination during any of the processing steps can occur (Zhang, et al., 2009). Several studies designed to investigate the presence of enteric pathogens in produce have shown that contamination with pathogenic *E. coli* and *Salmonella* occurs infrequently (Bohaychuk et al., 2009; Mukherjee et al., 2006; Koseki, et al., 2011). Systematic analysis for pathogens in produce lots or in the production environment is not likely to improve the safety of these products due to the sporadic nature of the contamination events. To better detect potential risk sources, there is a need of identifying sampling points which facilitate the evaluation of microbial contamination in the processing chain (Tomás-Callejas et al., 2011).

Assessing of microbial safety in RTE industries was traditionally based on end product testing to evaluate compliance with microbiological standards and quality guidelines implemented in the specific food system of the company. However, food processors are asked to voluntarily develop and implement a Food Safety Management Systems (FSMS) based on Good Manufacture Practices (GMP). Nevertheless, in the fresh-cut processing industry, there is still a need for the identification of suitable sampling points which facilitate the detection of contaminated produce. Several approaches for improving food safety of the fresh produce have been explored such as

the OmniFresh 1000 by Hanson Technologies, Inc. (US 7691602 B1). This system can detect small amounts of contaminated produce in large volumes of uncontaminated produce. The OmniFresh 1000 is based on the ultrafiltration of large volumes of process water from the washing tank coupled with an array biosensor for the pathogen detection.

It should be taken into account that contamination of leafy greens is considered as a 'rare' event, so direct pathogen screening is likely to be ineffective as an intervention. Additionally, the prohibitive cost, for smaller operations, and time consumption of the lengthy duration of the pathogen detection procedures make microbial indicators a good strategy to characterize microbial contamination in RTE products. *E. coli* spp. can have a dual purpose functioning as an indicator organism to verify Good Manufacturing Practices (GMP) and to some extent as an index organism to assess absence of significant faecal contamination (EFSA, 2014a). However, microbial indicators of faecal contamination do not necessarily reflect the input of enteric pathogens (EFSA, 2014a) and its validity as predictors of pathogens presence is controversial. The relationships/correlations between the presence or levels of a pathogen and an indicator are random, site-specific, or time-specific events (Payment & Locas, 2011). As a result, there is clearly no particular indicator that is suitable for all pathogens in all environments (Wilkes et al., 2009). Only few studies have focused on the correlation between the distribution of indicator microorganisms and the prevalence of foodborne pathogens in leafy greens at the processing level (Holvoet et al 2012). Considering the above, sampling at three fresh-cut produce companies as well as lab scale tests were performed to identify critical sampling points for the evaluation of microbial contamination of RTE products.

2. Materials & Methods

2.1 Sampling plan

Three fresh-cut processing companies were involved in this study. These companies followed the same standard processing operations for this type of produce (EU 854, 2004). The three companies used chlorine-based sanitizers to maintain the quality of the washing water. There were slight differences among the processing scheme of the selected fresh-cut companies such as the number of washing tanks and the production rate. In the case of companies 1 and 2, two washing steps were implemented (pre–wash and wash steps), while only one washing step was applied in company 3. The amount of processed baby spinach was similar in Companies 1 and 2 with an estimated volume of 450 kg/h and higher in company 3 with a value of 600 kg/h. The 3 fresh-cut processing companies belong to the main fresh-cut producers, which represent up to 80% of the Spanish production. The temperature of the three processing plants during the execution of this study was between 6-10 ºC. Each company processed RTE baby spinach during the working day, starting at 6 am and finishing around 3 pm. Samples were taken at three sampling times: the beginning (T1), in the middle (T2) and at the end of the working day (T3) (**Figure 6.1**). The sampling plan was carried out at three independent visits in different days for each processing company. Samples of fresh produce (i.e. raw material and end product), water (pre-wash water, wash water, rinse water and centrifuge effluent water) and surfaces (workers' plastic gloves, conveyor belts, centrifuge and weighing surfaces) were taken for analysis. At least 9 samples of produce (100 g each) were randomly taken from each sampling points. Water samples (4 L each) were collected into sterile bottles. For surface samples, sterile swabs were used for sampling 50 cm^2 of workers' plastic gloves

and equipment surfaces. Swabs were then immersed in 5 mL buffered peptone water (BPW). All the samples were transported to the laboratory in a covered container under refrigerated conditions (5-10 ºC), where further handling and microbial analysis were conducted within 2-12 h. A total of 879 samples have been analysed during this longitudinal assay.

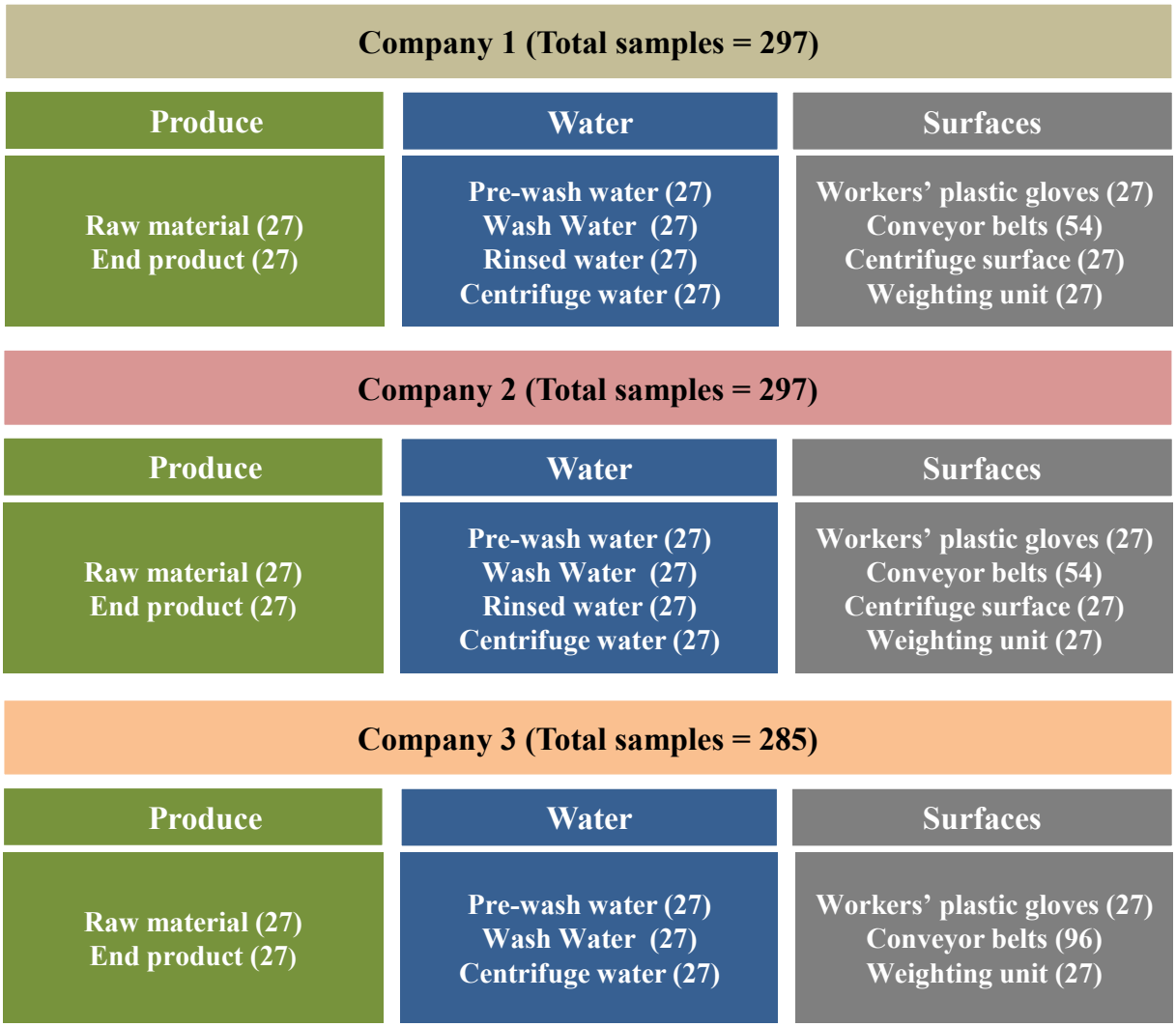

Figure 6.1. Sampling scheme followed in this study.

2.2. Microbial analysis

2.2.1 Pathogenic microorganisms

Presence or absence of STEC (*E. coli* O157:H7 and four shiga-toxin producing strains *E. coli* O26, O103, O111, and O145) and *Salmonella* spp. were determined in

produce and water samples (n=144) (Desroche et al. 2009; Holvoet et al., 2014a). Solid samples were homogenized for 1 min in 225 mL of BPW (AES Chemunex) and incubated for 18 ± 2 h at 37 °C for enrichment. Water samples (1 L each) were filtered and the filters incubated in 100 mL BPW at 37 °C for 18-20 h for enrichment. Fifty microliters of each enriched sample were used to extract and purify the bacterial DNA using a commercial extraction kit (Extraction Pack Food, Pall®, Port Washington, US) for *Salmonella*, STEC (O157, O26, O103, O111, and O145), and *Listeria* detection. Samples were analysed using the validated real-time PCR (RT-PCR) method of GeneDisc® Rapid Microbiology System (Pall®, Port Washington, US). In the case of a positive RT-PCR, isolation and confirmation of colonies was attempted. Before isolation, 1 mL of frozen (30% glycerol) enriched samples was subjected to a second non-selective enrichment in 10 mL of BPW (AES Chemunex) at 37 ºC for 18-24 h. For the confirmation of *Salmonella* spp. positive samples, the ISO 16140:2003 method (ISO, 2003) was used for further isolation of presumptive *Salmonella* spp. colonies. Presence of *Listeria monocytogenes* was assessed in produce samples (n=108) (Holvoet et al., 2014a).

2.2.2 Indicator microorganisms

A total of 879 samples were analysed for the identification of sampling points to detect microbial contamination. Counts of indicator microorganisms were monitored as previously described (Holvoet et al., 2014a). Generic *E. coli* was assessed as an indicator of faecal contamination and process hygiene microorganism. Total coliforms were tested in all sampling points. *Enterococcus* spp. were enumerated in water samples while *Enterobacteriaceae* were examined in surfaces and workers' plastic gloves. *E. coli,* total coliforms and *Enterococcus* spp. were enumerated in 100 mL water samples using cellulose nitrate membrane filters (0.45µM diameter, Microsart®, Sartorius,

Spain). Chromocult Agar (Merck, Darmstadt, Germany) was used for the enumeration of *E. coli* and total coliforms after incubation for 24 h at 37 °C in produce, water, and surface samples. *Enterococcus* were incubated on Slanetz-Bartley medium (Oxoid, UK, Europe) for 44 h at 37 °C. Then, filters were transferred to bile-aesculine-azide agar (Sigma Chemical, MO, US) for 2 h at 44 ºC. *Enterobacteriaceae* were enumerated using Violet Red Bile Glucose (Oxoid) and incubated for 24 h at 37 °C.

2.3 Validation of centrifuge effluent water as a sampling point for the evaluation of microbial contamination in the fresh-cut processing lines

Lab scale tests were performed to validate the suitability of the centrifuge effluent water as a sampling point for the enumeration of *E. coli* as an indicator microorganism. Baby spinach was harvested the day before and kept at 4 °C overnight. Five strains of generic *E. coli* (CECT 434, 515, 516, 533, 4972) were grown overnight in BHI at 37 °C. A cocktail was prepared mixing equal volumes from all the strains, centrifuged at 3200 *g* for 10 min and re-suspended in 0.1% BPW. Inoculum was diluted in beakers containing BPW 0.1% to a level of ≈4 log CFU/mL. Inoculated baby spinach was prepared by the immersion of the leaves for 1 min followed by storage overnight at 4 °C. Three replicates of 25 g of inoculated baby spinach were thoroughly mixed to obtain 300 g lots with a total of 5% of inoculated product. The same procedure was carried out for the un-inoculated samples as control. Samples (3 x 25 g) were taken before washing, and the remaining produce (225 g) was submitted to 1 min washing by hand agitation in 4.5 L of chlorinated tap water (25 mg/L free chlorine, 4 °C, pH 6.5 adjusted using citric acid). Then, baby spinach was rinsed by a tap water shower for 30 sec, and finally dewatered in a 10 L manual salad centrifuge for 1 min. Centrifuge

effluent water was collected and the volume measured. The residual chlorine was neutralized using sodium thiosulphate. Three samples (25 g each) of finished baby spinach were taken to assess the level of *E. coli* after washing and centrifugation. *E. coli* was enumerated in the water and in the finished product as explained above.

2.4 Statistics analysis

Three independent tests were performed in different days for each assay. For calculation and graphical presentation of the median and inter- quartile range (IQR) of microbial counts only positive samples (i.e., with numbers above the detection limit) were included. IBM SPSS Statistics 19 was used for statistical analysis. Except when stated otherwise, P values < 0.05 were considered statistically significant. Shapiro Wilk test was performed to assess the normality of the data (P > 0.05). Mann Whitney U test was used to determine the difference between the positive counts (non-zero microbial loads) of the indicators with respect to the sampling moment.

3. Results and discussion

3.1 Prevalence of pathogenic microorganisms in different sampling points of fresh-cut processing lines

L. monocytogenes was not found in any of the tested samples (data not shown). However, *L. monocytogenes* has been recovered before from the environment of fresh-cut processing plants (Zhang & Farber, 1996; Aguado et al., 2004), highlighting the importance of strict hygiene practices during processing. The current legislation for *L. monocytogenes* in RTE products is absence in 25 g at the end of processing and less than 100 CFU/g at the end of shelf life (EU, 2005). While cases of listeriosis involving

lettuce are few, eight recalls have been issued since 2010 for *L. monocytogenes*–contaminated leafy greens (Zeng et al., 2014). Furthermore, several studies have found this microorganism in RTE salads in different countries with different prevalence: 1.6% in the US (Lin et al., 1996), 0.6 and 3.2% in Brazil (Porto & Eiroa, 2001; Fröder et al., 2007), 2.3 and 4.8% in the UK (Sagoo et al., 2003; Little et al., 2007) and 10.2% in Chile (Cordano & Jaquet, 2009). However, in accordance with our results, in the few studies available, this pathogen was not found in RTE leafy greens (Farber et al., 1989; Guerra et al., 2001; McMahon & Wilson, 2001).

While pathogenic *E. coli* was not detected in any of the samples tested, *Salmonella* spp. were detected by multiplex RT-PCR analysis in 16 samples including the raw material and the end product as well as in the process wash waster and the centrifuge water **(Table 6.1**).

Table 6.1. Presence of pathogenic bacteria in samples taken at the three fresh-cut processing companies of baby spinach. RT-PCR: real time PCR.

Samples	***Salmonella* spp.**		***E. coli* O157:H7**		***E. coli* O26, O103, O111 & O145**	
	RT-PCR	Confirmed	RT-PCR	Confirmed	RT-PCR	Confirmed
Raw product	3/27	0/27	0/27	0/27	0/27	0/27
Wash water	5/45	0/45	0/45	0/45	0/45	0/45
Rinse water	0/27	0/27	0/27	0/27	0/27	0/27
Centrifuge water	4/26	2/26	0/26	0/26	0/26	0/26
End product	4/27	0/27	0/27	0/27	0/27	0/27

Within these 16 samples, only two of them were culture-confirmed, both of them obtained from the centrifuge effluent water. The absence of *Salmonella* spp. and pathogenic *E. coli* in the end product is in accordance with previous reports carried out in RTE salads in different countries such as Spain (Soriano et al., 2000), Ireland (McMahon & Wilson, 2001), the UK (Sagoo et al., 2001), Norway (Johannessen et al.,

2002; Loncarevic et al., 2005), Mexico (Johnston et al., 2005, 2006), Canada (Bohaychuk et al., 2009; Allen et al., 2013), the US (Phillips & Harrison, 2005), Italy (De Giusti et al., 2010), Singapore (Seow et al., 2012), Belgium (Holvoet et al., 2014a) and Iran (Avazpour et al., 2013; Jeddi et al., 2014).

For *Salmonella* spp. in RTE salads, Fröder et al. (2007) reported a prevalence of 3% in retailers in Brazil, while Abadias et al. (2008) reported a prevalence of 1.3% in Spanish supermarkets and Hara-Kudo et al. (2013) reported even a lower prevalence of 0.1% in Japan. Similarly, the US Food and Drug Administration performed a large produce survey (7646 samples) in retail markets and distribution centres finding that only 0.04% of samples tested positive for *Salmonella* spp., with no detection of *E. coli* O157:H7 (FDA, 2006). The situation is different in developing countries such as India that reported a prevalence of 33.3% in salad samples (Viswanathan & Kaur, 2001) and prevalence of 37% on produce in Egypt (Uyttendaele, et al., 2014). Higher *Salmonella* spp. prevalence in less-developed countries has been associated with the use of untreated wastewater for irrigation, contaminated with human faeces (Dreschel et al., 2010). Thus, with the exception of developing countries, the overall prevalence of *Salmonella* spp. on leafy greens is assumed to be low (< 1%) (EFSA, 2014a).

In our study *Salmonella* spp. were confirmed in two samples of effluent water from the centrifugation operation of the same company and day of sampling (T1 and T2) (**Table 6.1**). This fact suggests that the origin of the pathogen might be the raw material before washing, as the effluent water from centrifugation is the water located at the surface of the produce. Only a small number of studies have assessed the presence of foodborne pathogens in the processing facility for fresh-cut produce. Da Cruz et al. (2008) investigated the hazards involved in different processing operations of fresh-cut lettuce and reported high microbial load of indicator microorganisms but absence of

Salmonella spp. (n=30). A recent study carried out by Holvoet et al. (2012) investigated the degree of microbial contamination in the processing chain of fresh-cut lettuce without finding *Listeria monocytogenes* or *Salmonella* spp. in the product before and after processing (n=39). However, a negative result should be interpreted with caution due to the low number of samples analysed in the aforementioned studies and in ours, where the number of produce samples tested for pathogens was also low (n=54) (**Table 6.1**). Pilot-plant scale studies have been carried out in order to better understand the cross-contamination with pathogens that can occur during processing. Buchholz et al. (2012a) demonstrated that approximately 90% of the *E. coli* O157:H7 population on dip-inoculated leafy greens was shed in sanitizer-free water, with this pathogen also contaminating the product contact surfaces of a shredder, conveyor, flume tank, shaker table, and dewatering centrifuge to various degrees during processing. The same authors but in another pilot-plant scale study demonstrated that one contaminated batch of leafy greens (10^2-10^6 CFU/g *E. coli* O157:H7) can easily contaminate subsequent batches of previously uncontaminated product in a processing facility if an effective microbial intervention is not implemented (Buchholz et al. 2012b). Our results regarding pathogenic bacteria confirm that their sporadic presence in low levels hinders the attainment of meaningful data on their distribution and fate in processing lines.

3.2. Levels of generic *E. coli* and other microorganisms in different sampling points of fresh-cut processing lines

All the produce samples analysed in our study were negative for the presence of pathogenic bacteria, confirming that the contamination of leafy greens with pathogenic bacteria is considered a rare event in prevalence and frequency, expected to be below the practical probability of detection in the majority of circumstances. Thus, the direct

pathogen screening is ineffective and finding a positive is unlikely due to the relative low number of samples tested (n<100) and low contamination level. As a consequence, a number of studies use indicator microorganisms as a proxy for foodborne pathogens (Ailes et al., 2008; Ceuppens et al., 2014; Castro-Ibáñez et al., 2015a,b). In the present study all the baby spinach samples were negative for generic *E. coli* (**Figure 6.2**).

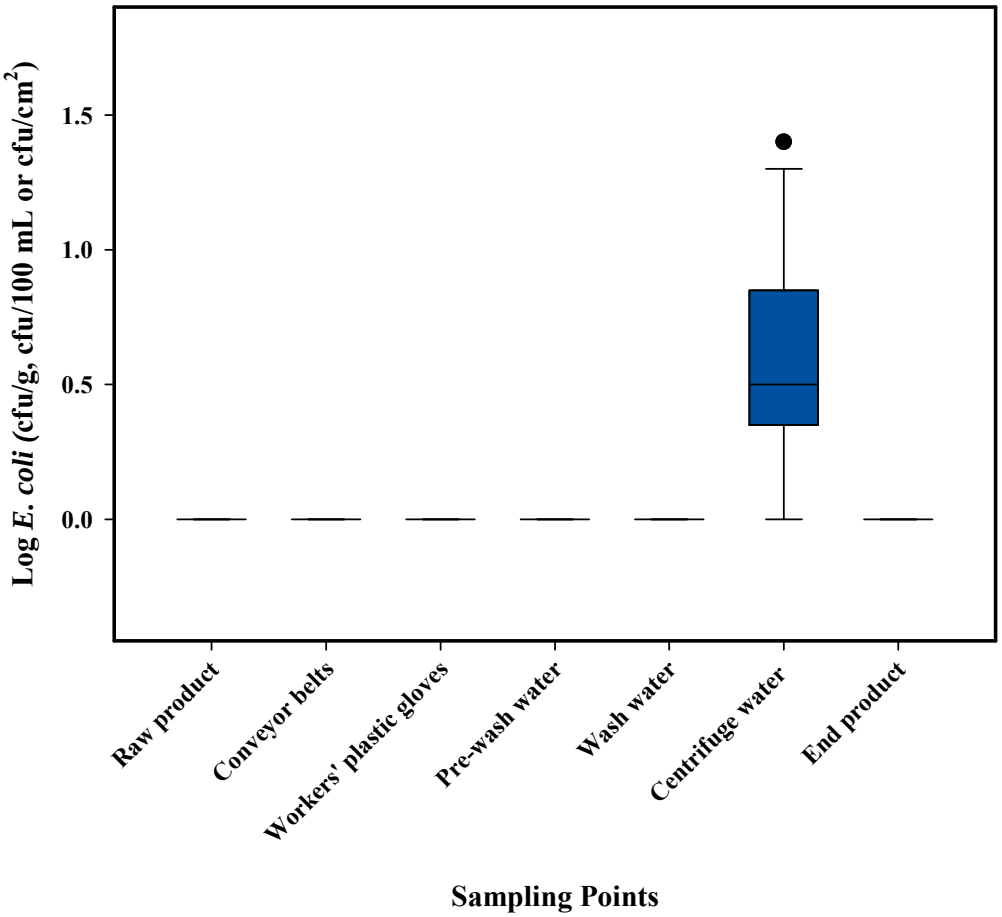

Figure 6.2. Boxplot representing *E. coli* counts in samples of produce (log CFU/g), water log CFU/100 mL and surfaces (log CFU/cm^2). In the boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest and lowest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile).

Prevalence of generic *E. coli* on RTE salads is presumed to be low (1%) (Sagoo et al., 2003). However a number of studies investigating the microbiological quality of RTE salads have reported higher prevalence in countries such as Spain (11.4%)

(Abadias et al., 2008), the US (16%) (Valentin-Bon et al., 2008) and the United Arab Emirates (20%) (Almualla et al., 2010). As in the case of pathogenic bacteria, higher prevalence of *E. coli* (>50%) has been observed in developing countries such as Argentina (64%) (Pellicer et al. 2002), Mexico (85%) (Castro-Rosas et al. 2012), Iran (69%) (Avazpour et al. 2013) and Egypt (100%) (Khalil et al., 2015).

Regarding the equipment surfaces and workers' plastic gloves, *E. coli* was not found in any of the samples tested (**Figure 6.2**). The results obtained contrast with those reported by Holvoet et al. (2012), who showed that *E. coli* could be found on conveyor belts and weighing surfaces in a Belgian fresh-cut industry, highlighting these facilities as potential sources for cross-contamination. The differences regarding *E. coli* levels could be due to the use of wash water sanitizers in Spain (Gil et al., 2009). Furthermore, the absence of *E. coli* in surfaces and workers' plastic gloves would be related with the good level of implementation of GMPs in the three companies, although this guideline is not mandatory it is frequently implemented in fresh-cut processing companies and covers all aspects of a processing environment from the design of a sanitary facility to rules forbidding jewellery on workers in order to prevent any type of contamination event (Garrett et al., 2003). Japanese researchers examining processing factories of RTE vegetables found three *E. coli* isolates corresponding to the floor of the processing rooms and the surfaces of workers' plastic gloves (Kaneko et al., 1999). Water used during processing of leafy greens has been identified as a potential risk source for cross-contamination with faecal indicator microorganisms and human enteric pathogens (Allende et al., 2008; Luo et al., 2011; Buchholz, et al., 2012b; Holvoet et al., 2012; Rodriguez-Lazaro et al., 2012; Shen et al., 2013; Holvoet et al., 2014a; Gil et al., 2015). When contaminated fresh-cut lettuce with *E. coli* is in the washing bath, a rapid transfer of this microorganism from the lettuce to the washing water occurred (López-Gálvez et

al., 2010a; Holvoet et al., 2014b). In the present study, the three processors had a reliable control of the water quality as they followed the European microbiological criteria for potable water in the washing tanks (absence of *E. coli* and Enterococci in 100 mL) (EU, 1998). Samples positive for *E. coli* were only found in water samples taken at the centrifugation operation (**Figure 6.2**). Furthermore, *E. coli* levels in centrifuge water significantly increased from the beginning until the end of the production day (Kruskal-Wallis test p=0.01) (**Figure 6.3**). These results, together with the confirmation of *Salmonella* spp. in two centrifuge water samples, highlighted centrifugation water as a critical control point for the detection of microbial contamination in the fresh-cut processing lines. According with these results, centrifuge effluent water could be used as a sampling point for the evaluation of microbial contamination of a lot (Tomás-Callejas et al., 2012). However, regarding *E. coli* numbers, it must be acknowledged that no specific confirmation tests were conducted to confirm taxonomic identity of *E. coli* in the enumerated counts.

Enterococcus spp., *Enterobacteriaceae* and total coliforms were also tested as indicator microorganisms. Results showed high levels of total coliforms (data not shown) and *Enterobacteriaceae* in all the sampling locations (**Table 6.2**).

These results are in accordance with Fröder et al. (2007) who found total coliforms and *Enterobacteriaceae* in 97.7% and 95.5% of RTE salads in Brazil and Sagoo et al. (2003) who found *Enterobacteriaceae* in all the tested RTE salads in the UK. Even though the populations of these indicators found in the present study were high (>10^3 CFU/g), these groups are common in raw vegetables and are not necessarily associated with faecal contamination (Brackett & Splittsoesser, 2001).

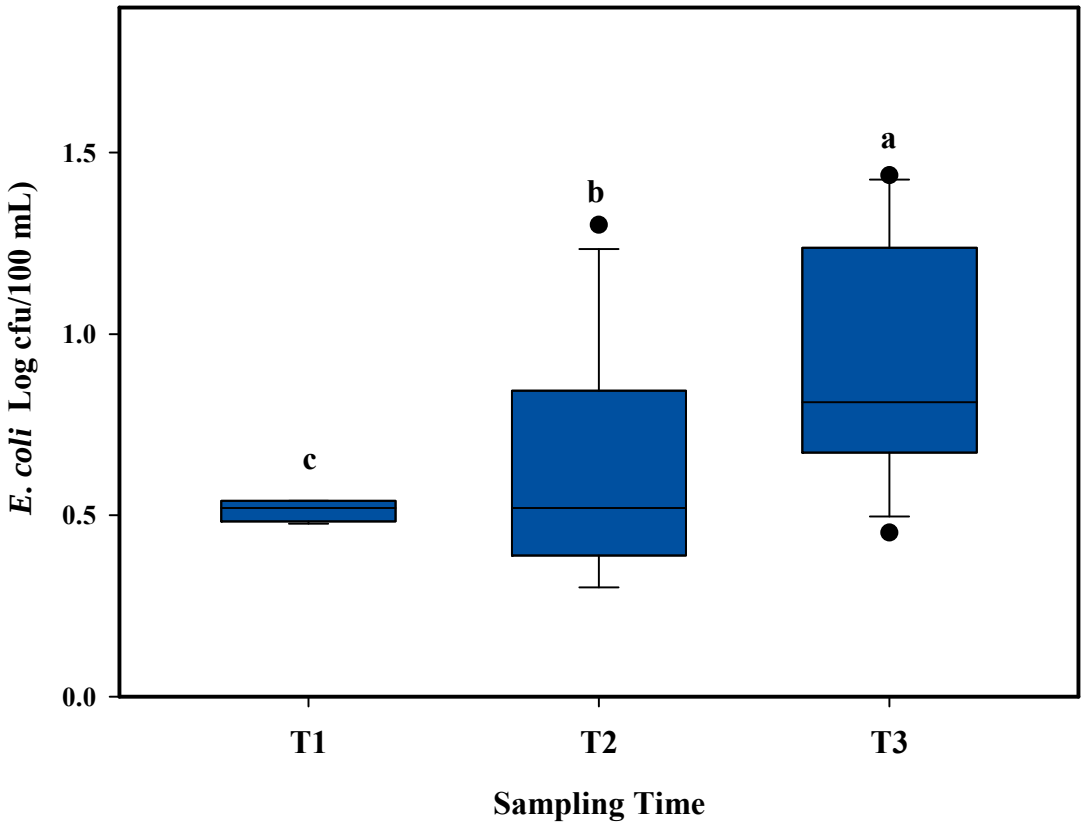

Figure 6.3. Boxplot representing *E. coli* counts (log CFU/100 mL) in centrifuge water samples at different moments of the working day. In the boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest and lowest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). Significant differences were determined by Kruskal–Wallis test ($P < 0.01$) and are represented with different letters.

On the other hand, all the water samples analysed were negative for *Enterococcus* spp. in accordance with Holvoet et al. (2012) who did not find enterococci in wash water of fresh produce processing industry.

Table 6.2. Counts and proportion of positive samples for *Enterococcus* spp. and Enterobacteriaceae in samples of produce (log CFU/g), water log (CFU/100 mL) and surfaces (log CFU/cm^2) taken at the beginning, (T1), in the middle (T2) and at the end of the working day (T3). Data are the median value and interquartile range (IQR) calculated using only samples with counts above the detection limit. NA: not analysed.

Sampling moment	Sample type	*Enterococcus* spp.		*Enterobacteriaceae*	
		Median	**IQR**	**Median** (positive samples/total)	**IQR**
T1	Raw material	NA	–	NA	–
	Pre -Wash water	<1 CFU/100 mL	–	NA	–
	Wash water	<1 CFU /100 mL	–	NA	–
	Rinse water	<1 CFU /100 mL	–	NA	–
	Centrifuge water	<1 CFU /100 mL	–	NA	–
	Workers' plastic gloves	NA	–	3.9 (27/27)	3.3–4.2
	Conveyor belts	NA	–	2.5 (37/64)	2.2–2.9
	Centrifuge surface	NA	–	3.0 (18/18)	2.6–3.2
	Weighting unit	NA	–	2.2 (6/27)	2.1–2.5
	End product	NA	–	NA	–
T2	Raw material	NA	–	NA	–
	Pre -Wash water	<1 CFU /100 mL	–	NA	–
	Wash water	<1 CFU /100 mL	–	NA	–
	Rinse water	<1 CFU /100 mL	–	NA	–
	Centrifuge water	<1 CFU /100 mL	–	NA	–
	Workers' plastic gloves	NA	–	4.1 (27/27)	3.9–4.6
	Conveyor belts	NA	–	3.4 (74/74)	3.8–4.4
	Centrifuge surface	NA	–	3.5 (18/18)	3.3–4.2
	Weighting unit	NA	–	3.3 (27/27)	2.7–3.4
	End product	NA	–	NA	–
T3	Raw Baby spinach	NA	–	NA	–
	Pre -Wash water	<1 CFU /100 mL	–	NA	–
	Wash water	<1 CFU /100 mL	–	NA	–
	Rinse water	<1 CFU /100 mL	–	NA	–
	Centrifuge water	<1 CFU /100 mL	–	NA	–
	Workers' plastic gloves	NA	–	4.0 (27/27)	3.7–4.3
	Conveyor belts	NA	–	3.7 (69/72)	4.0–4.4
	Centrifuge surface	NA	–	3.7 (18/18)	3.5–4.1
	Weighting unit	NA	–	3.5 (27/27)	3.2–4.0
	End product	NA		NA	–

3.3. Validation of centrifuge water as a sampling point for the evaluation of microbial contamination in the fresh-cut processing lines.

Detection of *Salmonella* spp. and generic *E. coli* in the centrifuge water suggested that the analysis of this water could replace end-product testing, allowing the identification of individual contaminated lots of produce. A lab scale study was performed with the purpose of gaining insight on the relevance of centrifuge water as a

sampling point for the immediate identification of a contaminated batch. Thus, after washing inoculated baby spinach, the levels of *E. coli* recovered from the centrifuge effluent water were enumerated. Median of *E. coli* level in baby spinach batches before washing was 1.2 log CFU/g. After washing and centrifugation, the median level of *E. coli* was 0.9 log CFU/g (**Figure 6.4**). Volume of centrifuge water ranged between 110 and 180 mL in the three repetitions carried out, and centrifuge effluent water was always positive for the presence of *E. coli*, although detected levels were low, ranging between 0.2 and 0.8 log CFU/100 mL, with a median of 0.4 log CFU/100 mL (**Figure 6.4**).

Buchholz et al. (2012b) also found pathogenic *E. coli* at quantifiable levels in the centrifuge water after inoculating baby spinach with low levels of a GFP-labelled strain of *E. coli* O157:H7. Tomás-Callejas et al. (2012) also found *E. coli* O157:H7 and *Salmonella enterica* spp. *Typhimurium* in centrifuge effluent water after lab-scale processing of inoculated red chard. In our experiment, the small number of *E. coli* cells found in centrifuge effluent water highlights the need for the filtration of higher volumes of centrifuge water in order to concentrate these indicator bacteria that is normally absent or in very low number. In primary production lettuce samples, Holvoet et al. (2014a) reported an *E. coli* spp. prevalence of 5% with a median of 1 log CFU/g. The lab-scale results provide evidence for the use of centrifuge water as a microbiological quality sampling point in fresh-cut processing lines.

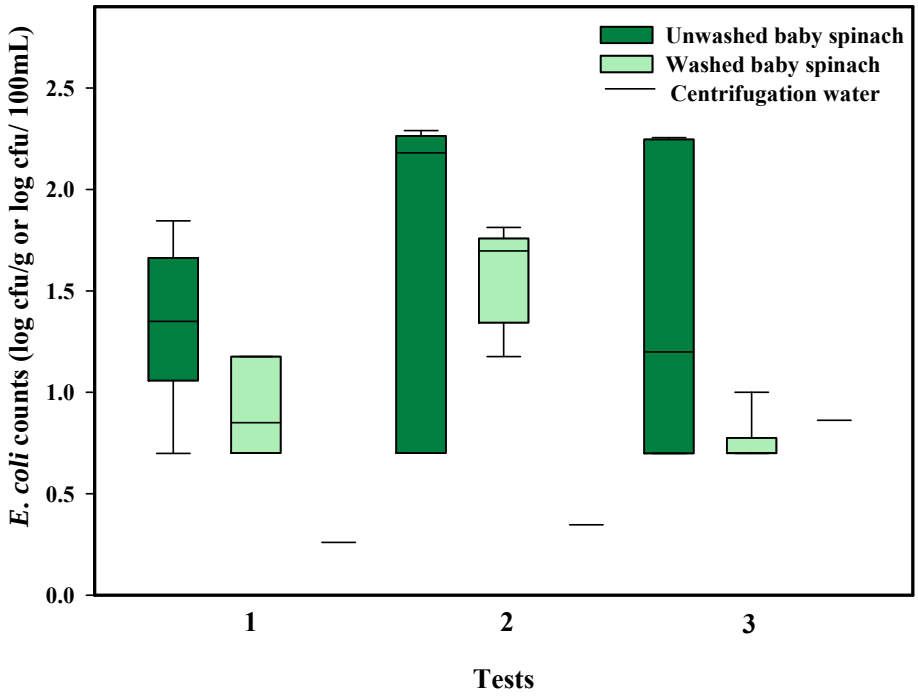

Figure 6.4. Boxplot representing *E. coli* counts on washed baby spinach (log CFU/g) and centrifuge water (log CFU/100 mL). In the boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median, whiskers show the greatest and lowest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile).

4. Conclusions

Our findings on the systematic sampling confirm that when FSMS based on GMP are strictly applied, pathogenic microorganisms and indicator microorganisms (*E. coli*) are seldom or not found in the processing facilities of the three Spanish fresh-cut produce companies studied. Pathogenic bacteria and generic *E. coli* were not detected in the end product. Nevertheless a negative result cannot guarantee product safety. Although generic *E. coli* was not detected in baby spinach, it was detected in the centrifuge water. Routine monitoring of the centrifugation effluent water for pathogens, *E. coli* or other indicator microorganisms may be an improved point of sampling to

determine the status of an individual operational shift or between points of testing for a given lot/lot definition but it still cannot be a critical control point as no technology may be applied to ensure pathogen elimination. Nevertheless, further research is needed to establish a correlation between *E. coli* levels in the centrifuge water and on the produce to make assumptions about the microbial safety of a lot when high indicator levels are detected in the centrifuge water.

Chapter VII.

Belgian and Spanish consumption data and consumer handling practices of fresh fruits and vegetables useful for further microbiological and chemical exposure assessment.

1. Introduction

Fresh produce can become contaminated with microbiological pathogens during primary production, in processing/packing, and/or during preparation (Ilic et al., 2012; EFSA, 2013; Gil et al., 2015). It has also been reported that complex contextual factors in fresh produce primary production, production processes, trade and logistic organization of the fresh produce supply chain, are affecting the food safety risk of fresh produce and derived food products (Ragaert et al., 2004; Tromp et al., 2010). The recent events on food contamination have enhanced the evaluation of risks associated with the food chain through the exposure assessment, a key step in risk assessment. Lately, risk assessment studies have been performed to calculate the exposure of consumers to pathogens by the consumption of fresh produce (e.g. Naguyen-The, 2012; Vinci et al., 2012; Ding et al., 2013; Sant'Ana et al., 2014). Also pesticide residues are still perceived by European consumers as being one of the most important food safety issues (Van der Linden et al., 2013). However, in the discussion of exceeding maximum residue limits of pesticides, an exposure calculation is necessary to investigate if human health is really endangered or not (EFSA, 2011c).

Different cultural habits, preparation and consumption patterns can be identified which may influence food safety risks. Within risk assessment, information about food consumption is necessary to calculate exposure of the population to a certain food safety hazard (Le Donne et al., 2011; Hoelzer et al., 2012). However, harmonized and detailed food consumption data are necessary to perform an exposure assessment. Usually, results of previous surveys or published papers are used for consumption data (EFSA, 2013). Though, there are several concerns related to the application of consumption data. First, the results of the survey should be recent and representative of the target

population. Specific populations such as children and elderly can be very vulnerable to specific chemical/microbial substances in food (Kroes et al., 2002; EFSA, 2009c; Hoelzer et al., 2012). Secondly, it is still hard to compare consumption data from different countries, due to different methods for data collection such as: the dietary assessment method e.g. 24-h recalls, food frequency questionnaires (FFQ) or via diaries, the number of days which are questioned, the sampling design and the quantification of portion sizes (Le Donne et al., 2011; EFSA, 2013). The status of the food is not always clearly indicated i.e. consumed raw or processed (e.g. cooked, grilled etc.). The lack of additional or confusing data on the status of processing was previously reported e.g. (Agudo et al., 2001), (EFSA, 2012) and (Soerjomataram et al., 2010). The portion sizes or units are not always clearly indicated. Selection of a specific commodity can also be problematic because there are sometimes other products within a product group, for example 'salads' contain also spinach intended to be cooked. And lastly, when addressing hazards with an acute impact on human health e.g. bacteria or viruses, the frequency of consumption and the portion sizes have to be known to calculate the exposure. While for hazards with chronic impacts as provoked by chemical hazards, the usual or daily intake is needed (De Boevre et al., 2013; Wiley et al., 2013).

To overcome these problems, a research project, EU Menu (EFSA, 2010b), has been launched to harmonize consumption data among European countries. However, since pre-standardization seemed impossible on an EU level, EU Menu has focused to a large extend on post-harmonisation of available intake data. Such post-harmonised data is not always optimal to calculate and compare dietary exposures between countries. In the US a comprehensive study was already performed to get a harmonized data set on fresh produce consumption (Hoelzer et al., 2012).

In the presented study, two European countries (Belgium and Spain) were evaluated regarding the suitability of existing data on the consumption of fresh fruits and vegetables in each country (Wolf et al., 2005; Verhoeff-Bakkenes et al., 2011; Le Donne et al., 2011). These two countries represent Northern versus Southern Europe and have two distinguished eating cultures. The latest Belgian national food consumption survey (conducted in 2004) used two independent 24-h recall interviews, in which all foods consumed within that time period were recorded. However, information on the frequency of consumption over a longer time period (e.g. one year) is not known for detailed food groups (e.g. vegetables being consumed raw) (Verhoeff-Bakkenes et al., 2010). These data must still be transformed into 'usual or daily consumptions' through specific software for exposure assessment to estimate for instance the risk on a yearly basis (Souverein et al., 2011). Therefore additional manipulation of the data is necessary (De Boevre et al., 2013; Willet et al., 2013). In the case of Spain, difficulties were found to identify the portion sizes or units (Wolf et al., 2005; Le Donne et al., 2011). It became clear that detailed information about actual consumption of different types of fresh produce and the corresponding handling practices conducted by consumers at home was still lacking. For that reason, consumption information of fresh produce consumed raw or minimal processing and potentially subjected to microbiological or chemical contaminations such as leafy greens, tomatoes, fresh herbs, strawberries, raspberries, other berries, pre-packed fresh-cut fruits and vegetables, fresh juices etc., in two European countries (i.e. Belgium, Spain), were collected based on a survey, using standardized dietary intake assessment methods, and further processed to applicable distributions for further acute and chronic exposure assessment calculations. In the survey, consumer handling practices such as storage method and time after purchase, refrigerator conditions and washing practices of

leafy greens were also included. These datasets can further be applied in microbiological or chemical exposure assessment linked to fresh produce consumption in Europe.

2. Materials & Methods

2.1 Development of questionnaire.

A questionnaire was developed to gain the necessary information of the selected case studies of fresh fruits and vegetables within European research project Veg-i-Trade (www.vegitrade.org). The questionnaire consisted out of three parts: (a) generic part containing information about the respondent (sex, age, level of education, etc.); (b) consumption part including data collection of consumed amounts of fresh fruits and vegetables and (c) consumer behaviour part including specific questions about handling fresh fruits and vegetables. The questionnaire was translated into Dutch, French and Spanish and evaluated for user friendliness by consulting non-researchers in both countries (i.e. friends, colleagues and family). Comments such as the layout of the questionnaire, limited choice of education and clarifying questions were implemented in the revised questionnaire. From this feedback, a final version of the questionnaire was obtained. The final version can be found in Annex 1 (http://www.foodscience.ugent.be/sites/default/files/LFMFP/article_fresh_fruits_and_vegetables/Annex1.pdf)

A quantitative portion (expressed in g or mL) was assigned to each portion size category for the different food items. The standard book on measures and weights was used to assign these quantitative portion sizes (Carrasco et al., 2010). A five point scale was applied to express the portions, where one stands for ‘product not eaten’ to five

which is the biggest portion. An overview of the portion size options and their corresponding weight in grams or centilitres is shown in **Table 7.1**. Respondents were asked to select the portion size that they most often consume.

The frequency of consumption was also asked in the food consumption questionnaire. Respondents had to indicate whether they generally consume the particular food item during a season or non-seasonal period throughout the year. If the answer 'seasonal' was selected, a numerical value of 0.25 (a quarter of the year) was assigned during data handling. 'Non-seasonal' received a numerical value of 1 (the food is consumed throughout the year). To indicate their frequency of consumption, the participants could choose from a present seven-point scale, where they could indicate how often they consumed the specific fruit or vegetable. The participants could choose between (a numerical value was assigned to these notions): Never (0), a few times per year (6 times per year), monthly (12 times per year), weekly (52 times per year), several times weekly (three times a week), daily and several times a day (three times a day).

2.2 Data collection of questionnaire.

The survey was distributed online via SurveyMonkey (https://www.surveymonkey.com/). In Belgium, an invitation to respond on the questionnaire was distributed via e-mail to students of Ghent University and personal mailing lists from researchers within Veg-i-Trade consortium in Belgium. An invitation to the survey towards the broader public was also made in magazines Test-Aankoop/Test-Achats (consumer organization in Belgium) and BodyTalk (magazine for broad public about health). The survey was also used in paper version for interviewing consumers during a commercial food fair in Ghent, mainly to gain some older consumers who might be difficult to reach via electronic mailing (November 2010). In

October 2011, additional data collection was done for the older consumers during presentation of 'food safety in historical context' for retired people. Actual data collection in Belgium was running from November 2010 (2th of November 2010) until October 2011.

In Spain, the online link was distributed by electronic mailing to students and staff of the University of Murcia, University of Cordoba, Technical University of Cartagena, University of Burgos, staff of the CSIC (Spanish National Research Council), personnel of Primaflor, Vega Mayor and Contariego (fresh-cut produce processing plants), consumer association in Murcia (http://www.consumur.org/) and personal mailing list of Veg-i-Trade researchers in Spain. The questionnaire was also sent to the online magazine EROSKY-CONSUMER (http://www.consumer.es/) (magazine for broad public about food and health). The survey was also used on paper version for interviewing consumers at different supermarkets as well as in food services from the University of Murcia (March 2011) in order to reach people who might be difficult to reach via electronic mailing. Actual data collection in Spain was running from February 2011 until August 2011. For the focus of research, only consumers between 18 and 65 years old were included in the dataset.

2.3 Data handling

The usual or daily intake of each food product consumed per day (expressed as g consumed/day) was calculated for each respondent by multiplying the seasonal conversion factor with the frequency and portion size of that commodity:

Then, the mean or average, median and standard deviation of usual consumption was calculated for all respondents for the different fresh fruits and vegetables. The

distribution of the acute consumption, being the combination of portion size and frequency of consumption, could be derived. The fractions of the respondents consuming a certain portion with a certain frequency were extracted via SPSS (Statistics Package for the Social Science, version 19) and tabulated in order to make a discrete distribution out of these data:

Discrete({a1 ; … ;ax} ;{b1 ; … ;bx}) (eq. 1)

with a = portion weight (e.g. portion (g)) and b = the corresponding number of consumers

Discrete({c1 ; … ;cx} ;{d1 ; … ;dx}) (eq. 2)

with c = consumption frequency (converted into numerical values) and d = the corresponding fraction of respondents

2.4 Quality control and cleaning of data in missing values and extreme values.

The resulting database was analysed using SPSS (Statistics Package for the Social Sciences, version 19). Missing or extreme values in the daily/acute consumption can be due to missing or extreme values in seasonality, frequency and/or portion size. To optimise the power, and as such the number of individuals available for statistical analyses, a search for the cause of the missing or extreme values was conducted and adjusted when possible. If the respondents have answered that they are not consuming a specific commodity, there is a chance that they are not filling in the portion question also. If the portion size is missing and the frequency of consumption is zero, a portion size of zero is inserted. In case of the frequency, an empty frequency is replaced by the

frequency which is most common in the completed frequencies by the other respondents. Because the frequency is strongly associated with the season, seasonality was also considered in the data cleaning. If seasonality was indicated, the missing frequencies were replaced by the most frequent frequency indicated for seasonal consumption by the other respondents. If non-seasonal consumption is indicated the missing frequency is replaced by the most frequent frequency of the non-seasonal frequencies.

This was the case for strawberries, where 753 missing values were present in the Belgian dataset. An additional dataset with only those data which are missing in the daily consumption of strawberries was made to investigate this problem in more depth. It was seen that in this case, the seasonal consumption was indicated but the frequency was not indicated anymore. The most given frequency for consumption of strawberries is 0.143 or a weekly consumption of strawberries in the season. If non-seasonal consumption is specified for strawberries, a frequency of 0.33 or monthly consumption of strawberries is indicated. After adapting the databases for missing values, a quality check on extreme values was conducted. The occurrence of extreme values in the daily consumption causes a large difference between the median and the average. Consuming extremely large quantities of a particular type of food is possible, but usually this extreme value is due to an error in the interpretation of the question by the respondent. As reported by (VCP, 2004), detailed Food Frequency Questionnaires (FFQs) that include many different food items/groups (like the one used in our survey) are often prone to over reporting, leading to extreme values, as subjects do not tend to sum up the portions and frequencies of consumption of the different food items/groups included in the food list. These extreme values were removed from the dataset and further discussed in results and discussion part of this paper.

Table 7.1. Weight or content of the corresponding portion sizes with respect to fruits and vegetables included in the survey.

Type of Fruit	Portion	Weight in g (* in cl)	Type of Vegetable	Portion	Weight in g (* in cl)
Apple	1. No consumption	0.00	Bell peppers	1. No consumption	0.00
	2. Two or three pieces	59.80		2. Two or three segments	68.00
	3. Half of the apple	86.70		3. Half of the pepper	99.70
	4. Entire apple	120.70		4. Whole pepper	165.40
	5. Two apples	245.40		5. Two peppers	334.58
Berries	1. No consumption	0.00	Cherry tomatoes	1. No consumption	0.00
	2. One spoon or ¼ of cup of 100g	14.20		2. From one to five pieces	41.70
	3. ½ of cup of 100g	56.60		3. Half of the container of 250 g	147.60
	4. ¾ of cup of 100g	84.68		4. ¾ of the container of 250 g	208.78
	5. Entire cup of 100g	112.90		5. Entire container of 250 g	250.00
Grapes	1. No consumption	0.00	Lettuce head	1. No consumption	0.00
	2. Five to seven grapes from a branch	24.80		2. Handful of lettuce head	43.10
	3. Half of branch	256.89		3. Half of a lettuce head	195.85
	4. ¾ of a branch	366.68		4. ¾ of a lettuce head	293.80
	5. Whole branch	486.23		5. Whole lettuce head	391.70
Pre-packaged fruit mixes	1. No consumption	0.00	Prepackaged mixed vegetables	1. No consumption	0.00
	2. One spoon of the mix	30.20		2. Handful	42.76
	3. Half of the mix	118.10		3. Half of a 125 g package	56.98
	4. Entire mix (250g)	248.60		4. Entire 125 g package	119.87
	5. More than 250 g of fruit mix	500.00		5. Entire 300 g package	286.93
Raspberries	1. No consumption	0.00	Prepackaged refrigerated mixed salads	1. No consumption	0.00
	2. ¼ of cup of 125g	25.23		2. Handful	11.90
	3. ½ of cup of 125g	60.33		3. Half of a 125 g package	49.70
	4. ¾ of cup of 125g	92.08		4. Entire 125 g package	108.76
	5. Entire cup (125 g)	118.67		5. Entire 300 g package	279.83
Smoothies and fresh juices in the refrigerator	1. No consumption	0.00	Fresh herbs	1. No consumption	0.00
	2. Half of 25 cl glass	12.50		2. Half of a teaspoon	0.60
	3. ¾ glass of 25 cl	18.75		3. Entire teaspoon	0.80
	4. Overall 25 cl glass	25.00		4. Half of a table spoon	1.30
	5. More than one glass	33.00		5. Entire table spoon	1.70
Strawberries	1. No consumption	0.00	Tomatoes	1. No consumption	0.00
	2. ¼ of cup of 250g	59.80		2. Two or three segments	60.90
	3. ½ of cup of 250g	86.70		3. Half of the tomato	80.90
	4. ¾ of cup of 250g	120.70		4. Whole tomato	149.60
	5. Entire cup (250 g)	245.40		5.Two tomatoes	310.26

*Daily consumption per respondent (g/day) = seasonal conversion (converted in numerical value) x frequency (converted into numerical value) x portion size (g or mL).

3. Results and discussion

For Belgium respectively 1722 respondents and 714 respondents from Spain answered the questionnaires (before data cleaning, **Table 7.2**). The majority were women, respectively 72.7% in Belgium and 62.5% in Spain. A differentiation is made between occupation i.e. student, working, at home or retired. The major respondent group included active persons, working, respectively 57.8% in Belgium and 79.3% in Spain. In Belgium also approximately 1/3 were students and a minor group of retired people were reached (5.5%) (**Table 7.2**). Due to the convenient sampling design used, our study population cannot be considered as representative for the total population in Spain/Belgium. It is for instance likely that people with an important interest in diet/nutrition are overrepresented in this survey. The over-representation of female respondents might for instance the effect the outcomes of our study because women are known to consume more healthy food including fresh produce (e.g. recently published for Spain with university students (Franz et al., 2010), in cross-European studies (Agudo et al., 2002; Wolf et al., 2005). Women are also presumed to be more aware of good kitchen practices compared to men (e.g. demonstrated in US (Li-Cohen & Bruhn, 2002) and in Belgium (Sengun, 2013)). Due to the applied acquisition pathways of respondents e.g. via university, via magazines, a higher socio-economic status is achieved on our sample. It was reported in several countries that population group with a higher socio-economic status is consuming more fresh produce (e.g. in US (Wolf et al., 2005) and (CDC, 2010c), in UK (Dibsdall et al., 2003), in Norway (Kvaavik et al., 2014)). Although related to consumers practices and treatment of fresh produce in home kitchens conflicting information is yet available (e.g. lower income households have reported better practices in fresh produce in US study of 2002 (Li-Cohen & Bruhn, 2002), while other studies demonstrated higher education level with corrected consumer

practices (e.g. Wolf et al., 2005). Due to the non-representative status of our study, our findings will tend to demonstrate a better situation both in consumption and consumer handling practices than we could expect when a full representative sample was selected for the whole Spanish/Belgian population. The population bias in Spain and Belgium is similar so the comparison between the two countries in this study is possible.

Table 7.2. Composition of respondents (men and women from the age group of 18 to 65 years in function of occupation) before data cleaning for Belgium and Spain

Country	Occupation	Men		Women		Total	
		Number	Percentage	Number	Percentage	Number	Percentage
Belgium	Student	123	7.2%	373	21.6%	496	28.8%
	Worker	288	16.7%	708	41.1%	996	57.8%
	At home	34	2.0%	101	5.9%	135	7.9%
	Retired	24	1.4%	71	4.1%	95	5.5%
	Total	469	27.3%	1253	72.7%	1722	100.0%
Spain	Student	39	5.5%	36	5.0%	75	10.5%
	Worker	209	29.3%	357	50.0%	566	79.3%
	At home	20	2.8%	53	7.4%	73	10.2%
	Total	268	37.5%	446	62.5%	714	100.0%

3.1 Data cleaning.

Figure 7.1 shows the boxplot for fresh herbs and leafy greens consumption expressed as g/day for the Belgian and Spanish dataset respectively without removing the extreme values. Boxplots display the variation in the consumption data and extreme outliers are plotted as individual points. It is often very difficult to determine where the boundary lies between extreme outliers calculated via SPSS and what is a non reliable answer by the respondent. As is known, these high percentiles e.g. 99% percentile in the consumption data can be important if exposure assessment studies are performed (e.g. in chemical risk assessment studies (Wolf et al., 2005; De Boevre et al., 2013) or

microbiological calculations (Franz et al., 2010; Piealaat et al., 2014). Therefore, it was decided to use the Belgian food consumption data as a reference and to limit these extreme daily consumptions when exceeding the maximum portion size reported by the Belgian national food consumption survey. In this latter case, the portion was replaced by the maximum portion size reported in the Belgian food consumption survey in our consumption database (De Vriese et al., 2004; Verhoeff-Bakkenes et al., 2010). This can be seen as an upper limit since the data of the Belgian national food consumption survey were obtained from a sample of two non-consecutive one day 24-h dietary recall data in a nearly representative sample of the Belgian population.

The same maximum values are also used for the Spanish data, because no detailed information of a food consumption database is available for Spain. The portion size applied for the removal of the extreme values based on the Belgian national food consumption survey and applied for the Belgian and Spanish data were: for leafy greens 200.0 g (approx. ½ lettuce head), for fresh herbs 35.8 g, for strawberries 470.0 g (approx. 2 packages of 250g), for apples 531.3 g (approx. 4 apples), for berries 266.0 g (approx. 1 package of 250g), for grapes 950.0 g (approx. 2 branches), for raspberries 296.0 g (approx. 1 package of 250 g), for tomatoes 570.0 g (approx. 4 tomatoes or 50 cherry tomatoes), for bell peppers 148.0 g (approx. one fruit), for pre-cut leafy greens 200.0 g, for mixed vegetables 398.0 g, for fresh juices 500.0 g and for fruit mixes 602.0 g respectively. This approach could be discussed surely for the Spanish respondents because Belgian consumption data were applied for limiting of their extreme outliers. However, when investigating in detail for leafy greens and fresh herbs. Twenty-six values had to be removed from the Spanish dataset with a consumption ranging between 220g and 880g of leafy greens and no values for fresh herbs consumption.

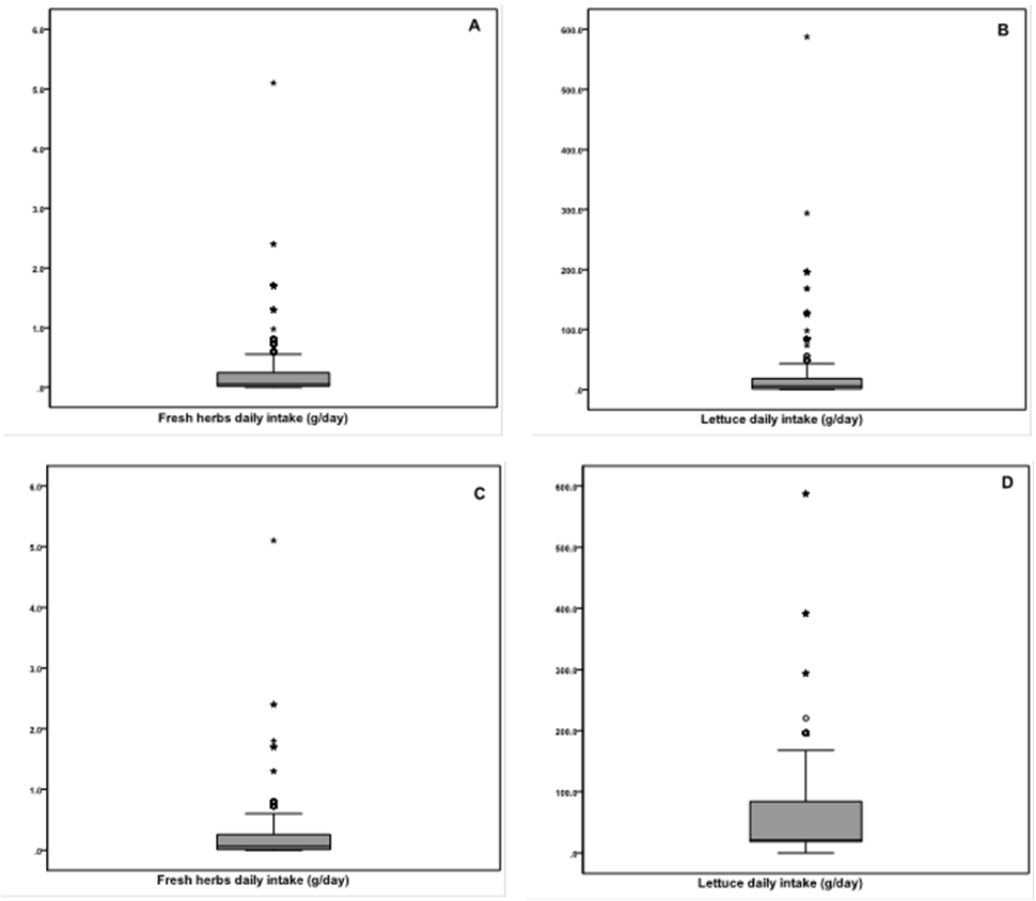

Figure 7.1. Boxplot of consumed quantity (daily consumption in g/day) for fresh herbs (A Belgium, C Spain) and lettuce head (B Belgium, D Spain), without data cleaning presenting (extreme) outliers (* represents an extreme outliers as a value more than three times the interquartile range from a quartile and ° is used to mark other outliers with values between 1.5 and 3 box lengths from the upper or lower edge of the box).

Therefore, it was decided that these extreme outliers are not representative for the Spanish consumption and we could indeed apply the Belgian national consumption as a cut off value for extreme outliers in our database. Finally, a last correction on age was conducted by excluding respondents younger than 18 and older than 65 years. The data cleaning has led to 583 retained respondents for Spain and 1605 for Belgium for calculating acute and daily consumption distributions. However, some variation in number of consumers can occur because not all respondents are consumers of all included commodities.

3.2 Consumption data expressed as usual daily intake (g/day).

The distributions of the daily consumption of the different types of fresh produce are illustrated in **Table 7.3** for the Belgian data and **Table 7.4** for the Spanish data, with indication of mean, median, standard deviation, and percentiles (based upon the total database, including consumers and non-consumers). For most products, median is different from mean and a large standard deviation is obtained. The major reason for this is because even after removal of extreme outliers, the distribution of the data is not normal, large tail to the right, left skewness in the distribution, which indicates that many consumers eat small portions and few consumers eat high portions (e.g. comparison of median and P95 and maximum). This trend can be exemplified with the case of lettuce consumption in Belgium: P50 is 6.16 g/day while P95 is 84.02 g/day and maximum is 587.55 g/day (**Table 7.3**). As mentioned before, consumers in these high percentiles may be subjected to higher exposure of both microbiological and chemical hazards (e.g. De Boevre, et al., 2013; Willet et al., 2013; Pielaat et al., 2014). The ranking of fruits consumed in Belgium and Spain based upon the median daily consumption (g/day) is: apple >> strawberry >> grapes >> berries & raspberry. The ranking of vegetables consumed in Belgium and Spain on basis of the median daily consumption (g/day) is: tomatoes >> leafy greens >> bell pepper >> fresh herbs. The median of apple consumption is 32 g/day both in Spain and Belgium. If an apple of 220 g is considered, then the daily consumption is approx. 1/7 of an apple. The median for grape consumption is 4.11 g/day corresponding with approx. 1 grape per day for Belgium. While in Spain a lower median of 3.55 g/day is calculated. The median for strawberry consumption in Belgium is 5.7 g/day corresponding with approx. 1 to 2 strawberries, while in Spain it is only 0.64 g/day.

Table 7.3. Distribution of daily consumption (g/day) in Belgium (after data cleaning) for respondents between 18 and 65 years old and fraction of non-consumers (n= 1605)

PRODUCT	MEAN	MEDIAN (P50)	σ	MIN.	MAX.	5%	10%	25%	75%	90%	95%	% non-consumers
Leafy greens/ lettuce head	15.88	6.16	35.05	0	200.00*	0.36	0.69	1.54	18.49	42.00	84.02	2.36
Fresh herbs	0.20	0.06	0.34	0	5.10	0.0	0.0	0.02	0.24	0.60	0.73	17.20
Strawberries	14.98	5.70	28.07	0	470.00*	0.64	1.29	2.66	13.35	34.52	70.18	2.23
Raspberries	2.80	0.72	5.91	0	118.67	0.0	0.0	0.28	2.88	8.41	14.32	18.19
Berries	4.71	0.47	12.72	0	254.04	0.0	0.0	0.12	2.03	12.11	24.24	17.53
Grapes	22.90	4.11	57.88	0	770.67	0.10	0.40	0.82	13.11	52.44	110.21	3.68
Apples	94.80	32.88	52.60	0	531.30*	1.21	1.90	8.22	98.63	229.90	229.90	2.56
Tomatoes[a]	39.94	21.39	57. 6	0	570.00*	0.67	2.67	8.67	44.37	133.10	149.60	3.94
Bell Pepper	11.73	5.46	18.56	0	148.00*	0.0	0.56	2.24	14.26	23.65	42.77	7.49
Lettuce mix	1.84	0.20	7.08	0	200.00*	0.0	0.0	0.0	1.64	3.89	7.11	27.97
Vegetables mix	1.37	0.0	4.18	0	51.42	0.0	0.0	0.0	0.91	3.80	6.11	52.00
Fruit juice	0.96	0.0	4.48	0	99.00	0.0	0.0	0.0	0.4	1.09	3.57	57.58
Fruit mix	0.71	0.0	3.89	0	88.57	0.0	0.0	0.0	0.0	1.89	3.98	81.35

[a] tomatoes include cherry tomatoes, *Maximum values are adapted due to cut off as data cleaning step based on Belgian consumption data.

Table 7.4. Distribution of daily consumption (g/day) in Belgium (after data cleaning) for respondents between 18 and 65 years old and fraction of non-consumers (n= 1605)

PRODUCT	MEAN	MEDIAN (P50)	σ	MIN.	MAX.	5%	10%	25%	75%	90%	95%	% non-consumers
Leafy greens/lettuce head	55.59	21.00	62.00	0	200.00*	1.31	4.62	18.49	84.02	195.85	195.85	1.26
Fresh herbs	0.19	0.06	0.80	0	5.10	0.0	0.0	0.01	0.25	0.56	0.73	12.21
Strawberries	2.54	0.64	7.39	0	80.47	0.16	0.16	0.64	1.29	4.28	11.51	1.80
Raspberries	0.43	0.0	2.30	0	39.56	0.0	0.0	0.0	0.13	0.66	2.88	25.49
Berries	1.02	0.0	5.84	0	112.90	0.0	0.0	0.0	0.23	1.86	6.09	25.31
Grapes	22.90	3.55	53.65	0	486.23	0.0	0.01	0.40	8.48	110.21	110.21	3.59
Apples	78.11	32.88	93.60	0	531.30*	0.92	2.50	8.22	98.63	229.90	229.90	0.72
Tomatoes[a]	104.31	64.18	105.30	0	570.00*	8.67	16.04	34.71	149.60	310.26	310.26	0.18
Bell Pepper	29.88	17.74	32.60	0	148.00*	0.23	2.24	7.29	42.77	70.96	99.70	2.51
Lettuce mix	15.61	5.11	30.99	0	200.00*	0.0	0.0	0.20	21.32	46.66	49.70	9.69
Vegetables mix	17.13	3.96	38.82	0	359.61	0.0	0.0	0.23	18.34	42.76	56.98	11.13
Fruit juice	6.23	0.86	10.29	0	99.00	0.0	0.0	0.10	10.73	25.00	25.00	10.41
Fruit mix	14.37	0.0	46.85	0	602.00*	0.0	0.0	0.0	3.90	50.66	106.65	25.67

[a] tomatoes include cherry tomatoes, *Maximum values are adapted due to cut off as data cleaning step based on Belgian consumption data.

Therefore, for the included fruits it can be derived that the usual consumption is lower in Spain compared to Belgium, except for the highest consumed commodity apple, where a similar median consumption is calculated.

The median for tomato consumption is 21.39 g/day corresponding with approx. 1/6 of a tomato in Belgium, while in Spain a much higher consumption with a median of 64.18g/day is calculated. The median for leafy greens consumption is 6.16 g in Belgium. Also for leafy greens, a higher Spanish consumption with median of 21 g/day is found. One handful of leafy greens is weighing 43 g, so on a daily basis 1/7 of a handful of leafy greens is eaten in Belgium and ½ of handful of leafy greens in Spain. The median for bell pepper consumption is 5.46 g/day in Belgium while 17.74 g/day in Spain, a whole bell pepper weighs approx. 165 g. For the included vegetables, the usual intake of the Spanish consumers is clearly higher compared to the Belgian consumers. Minimally processed products consumption in Belgium based upon the mean and median are only consumed in very small amounts (e.g. pre-cut leafy greens, pre-cut vegetable mix, juices and fruit mix) and non-consumers are majority of the Belgian respondents (**Table 7.3**). However, an emerging market for these convenience foods was reported in 2003 (Rijgersberg et al., 2010). In Spain, a higher consumption of these convenience foods was calculated **(Table 7.4**).

3.3 Consumption data expressed as acute intake

Also another representation of the collected data could be derived expressing the acute intake by portion and frequency of consumption. Therefore, the fractions of the respondents corresponding with a certain frequency and portion size consumed were extracted from the dataset with SPSS. An example for lettuce head is given in **Table 7.5.** These tables were elaborated for all other included commodities being strawberries,

other berries (e.g. raspberries, red berries), grapes, apples, tomatoes, bell peppers, prepacked leafy greens, packed vegetable mixes, fresh juices and smoothies and ready-to-eat fruit salads (Annex 2) (*http://www.foodscience.ugent.be/sites/default/files/LFMFP/article_fresh_fruits_and_vegetables/Annex2.pdf)*. This information can be further applied in acute risk assessment calculations as in the frame of microbiological risks e.g. *Salmonella* on leafy greens. An example of how these data can be transformed towards a discrete distribution is given in **Table 7.6** for lettuce head, where first the discrete distribution is made per consumption frequency (expressing the fraction of the consumers consuming a certain portion size) both for seasonal and non-seasonal consumers, based on equation 1. In a next step, the combination is made between consumption frequency and the portions (based on equation 2) and finally, seasonal and non-seasonal consumers are taken together to have the acute consumption of total consumers. Such an approach can be further incorporated in probabilistic risk assessment software for example in @Risk (Pallisade, U) in combination with concentration data of the hazard to calculate the risk of exposure. An example of microbiological risk assessment is (Sant'Ana et al., 2014) in which such an approach was applied for the consumption frequency (portion size were based on assumptions in this specific reference).

However, as consumption data are not always available, consumption data and/or consumption frequency of other countries/situations is often used as assumption. Official intake surveys are mostly presented as '*grams of produce per capita per kilogram of body mass*' or '*daily consumption'*. Also, when it comes to acute microbiological risk assessment, the average consumption over time is less relevant than the portion (e.g. Kroes et al., 2002; Willet, 2013). This is the case e.g. in risk assessment studies on fresh produce and use of greywater as irrigation source (Oron et

al., 2010) and the evaluation of irrigation water in home produced lettuce in Australia (Barket et al., 2013).

Table 7.5. Frequency and portion consumed by seasonal and non-seasonal consumers of head lettuce in Belgium and Spain.

Consumption of Lettuce		Belgium (n = 1605)		Spain (n = 583)	
		Seasonal (543 or 45%)	Non Seasonal (663 or 55%)	Seasonal (42 or 8%)	Non Seasonal (510 or 92%)
Few times a year (defined as 6 times per year)	Handful of lettuce (43.10 g)	24 or 4%	34 or 5%	2 or 5%	7 or 2%
	Half of a lettuce head (195.85 g)	3 or 1%	5 or 1%	0 or 0%	1 or 0%
	¾ of a lettuce head (293.80 g)	0 or 0%	1 or 0%	0 or 0%	0 or 0%
	Whole lettuce head (391.70 g)	0 or 0%	0 or 0%	1 or 2%	0 or 0%
	Total	27 or 5%	40 or 6%	3 or 7%	8 or 2%
Monthly	Handful of lettuce (43.10 g)	53 or 10%	137 or 21%	2 or 5%	13 or 3%
	Half of a lettuce head (195.85 g)	10 or 2%	31 or 5%	0 or 0%	1 or 0%
	¾ of a lettuce head (293.80 g)	0 or 0%	5 or 1%	0 or 0%	2 or 0%
	Whole lettuce head (391.70 g)	0 or 0%	2 or 0%	0 or 0%	1 or 0%
	Total	63 or 12%	175 or 27%	2 or 5%	17 or 3%
Weekly	Handful of lettuce (43.10 g)	232 or 43%	211 or 32%	7 or 17%	42 or 8%
	Half of a lettuce head (195.85 g)	51 or 9%	65 or 10%	1 or 2%	8 or 2%
	¾ of a lettuce head (293.80 g)	8 or 1%	10 or 1%	0 or 0%	4 or 0%
	Whole lettuce head (391.70 g)	4 or 1%	1 or 0%	2 or 5%	7 or 2%
	Total	295 or 54%	287 or 43%	10 or 24%	61 or 12%
Three times a week	Handful of lettuce (43.10 g)	90 or 16%	88 or 13%	10 or 24%	157 or 31%
	Half of a lettuce head (195.85 g)	26 or 5%	37 or 6%	4 or 10%	69 or 13%
	¾ of a lettuce head (293.80 g)	8 or 1%	7 or 1%	3 or 7%	21 or 4%
	Whole lettuce head (391.70 g)	3 or 1%	1 or 0%	1 or 2%	15 or 3%
	Total	127 or 23%	133 or 20%	18 or 43%	262 or 51%

Daily	Handful of lettuce (43.10 g)	24 or 5%	16 or 3%	2 or 5%	81 or 16%
	Half of a lettuce head (195.85 g)	7 or 1%	8 or 1%	4 or 10%	41 or 8%
	¾ of a lettuce head (293.80 g)	0 or 0%	1 or 0%	1 or 2%	9 or 2%
	Whole lettuce head (391.70 g)	0 or 0%	1 or 0%	1 or 2%	12 or 2%
	Total	31 or 6%	26 or 4%	8 or 19%	143 or 28%
Three times a day	Handful of lettuce (43.10 g)	0 or 0%	1 or 0%	0 or 0%	11 or 2%
	Half of a lettuce head (195.85 g)	0 or 0%	1 or 0%	1 or 2%	6 or 2%
	¾ of a lettuce head (293.80 g)	0 or 0%	0 or 0%	0 or 0%	1 or 0%
	Whole lettuce head (391.70 g)	0 or 0%	0 or 0%	0 or 0%	1 or 0%
	Total	0 or 0%	2 or 0%	1 or 2%	19 or 4%
Consumers		1206 or 75%		552 or 95%	
Non-consumers		43 or 3%		5 or 1%	
Not answered		356 or 22%		26 or 4%	

Assumptions on consumption portion (e.g. 100 g iceberg lettuce in (Palumbo et al., 2007)), serving size (e.g. between min 25 g, most likely 50 g and max 75 g of ready to eat vegetables in Brazil in (Sant'Ana et al., 2014), average consumption (e.g. 73 g of a salad portion in the Netherlands in (EFSA, 2013), and/ or frequency (e.g. in 120 day exposure in one year) for different commodities by risk assessment on *Cryptosporidium* and *Giardia* in (Nguyen-The, 2012) have been used. Consumption habits can be highly cultural dependent, e.g. a serving size of 85 g of cut leafy greens was used in a QMRA (Davidson et al., 2013) as a representative portion size for United States, while in Australia it was defined as 23.8 g of lettuce (Barker et al., 2013) and a consumption portion of 10-12 g of raw salads was representative for the consumption pattern in Ghana (Seidu et al., 2008). In this paper, even in closer living regions such as

consumers in Spain and Belgium, a considerable difference in consumption frequency and portion depending on the type of commodity was found.

Table 7.6. Discrete distribution constructed for acute exposure assessment from consumption of head lettuce in Belgium (n=1206)

Expression		Function
Portion weight - Seasonal (discrete function portion weight and fraction of respondents based on eq. 1)		
Portion few times a year	Cell 1	Discrete({43,1;195,85;293,8;391,7};{24;3;0;0})
Portion monthly	Cell 2	Discrete({43,1;195,85;293,8;391,7};{53;10;0;0})
Portion weekly	Cell 3	Discrete({43,1;195,85;293,8;391,7};{232;51;8;4})
Portion few times a week	Cell 4	Discrete({43,1;195,85;293,8;391,7};{90;26;8;3})
Portion daily	Cell 5	Discrete({43,1;195,85;293,8;391,7};{24;7;0;0})
Portion several times a days	Cell 6	none
Portion weight - Non seasonal[a] (discrete function portion weight and fraction of respondents based on eq. 1)		
Portion few times a year	Cell 7	Discrete({43,1;195,85;293,8;391,7};{34;5;1;0})
Portion monthly	Cell 8	Discrete({43,1;195,85;293,8;391,7};{137;31;5;2})
Portion weekly	Cell 9	Discrete({43,1;195,85;293,8;391,7};{211;65;10;1})
Portion few times a week	Cell 10	Discrete({43,1;195,85;293,8;391,7};{88;37;7;1})
Portion daily	Cell 11	Discrete({43,1;195,85;293,8;391,7};{16;8;1;1})
Portion several times a days	Cell 12	Discrete({43,1;195,85;293,8;391,7};{1;1;0;0})
Frequency of consumption (discrete function frequency of consumption and fraction of respondents based on eq. 2)		
Portion seasonal	Cell 13	Discrete(C1:C6;{27;63;295;127;31})
Portion non seasonal	Cell 14	Discrete(C7:C12;{40;175;287;133;26;2})
Combined consumers seasonal and non seasonal		
Portion Seasonal – non seasonal	Cell 15	Discrete(C13:C14;{543;663})

[a]Based on the data in Table 5.

[b]Discrete function portion weight and fraction of respondent based on equation 1.

[c]Discrete function frequency of consumption and fraction of respondents based on equation 2.

3.4 Strengths and limitations of the proposed approach in data collection, cleaning and application of the data towards chronic or acute exposure

Strengths of the presented study are the fact that two distinguished eating cultures were compared (Northern versus Southern Europe) where clear differences in consumption frequency and portion size were found in terms of acute and daily consumption. Also, comparable and standardized dietary intake assessment methodologies, used in the two countries, have made it possible to compare eating habits and also to merge the information into risk assessment studies. Standardized data handling procedures were used in both countries, again leading to the potential application in both acute and chronic exposure assessments.

Limitations of the represented study can be the fact that Food Frequency Questionnaires (FFQ) are not ideal to estimate acute dietary exposure (24-h recall or dietary record method are better for assessing acute exposures), though FFQ is valid and represents an optimal method for estimating chronic exposure of particular food groups or components (e.g. raw fruits and vegetables in this manuscript). Also a representative sample of population was not obtained. Over-representation of female respondents and higher socio-economic status could influence our findings, where a higher consumption and better consumer practices can be expected in our respondent group. And finally, the median was used to replace missing values, though sensitivity analyses have shown similar results when using mean (data not shown).

3.5 Consumer handling practices

The results of the behaviour of consumers after purchase of the fresh vegetables and fruits, are represented in **Table 7.7** (storage period after purchase) and **Table 7.8** (storage method). The duration of the storage time is in general shorter in Belgium compared to Spain: 8/11 included commodities are stored between 1-3 days after purchase in Belgium while 5/11 in Spain. Apples seem to be kept the longest of all considered fruits and vegetables. In terms of storage method, refrigerator conditions are most frequently applied in Belgium, where only fresh herbs, apples and grapes were more stored at room temperature. In Spain, all commodities were stored in refrigerated conditions by the majority of the respondents (**Table 7.8**). The duration and temperature conditions between purchase and consumption can play a role in potential survival, decline or growth of certain pathogens on (fresh-cut) produce and will influence the outcome of microbiological risk assessment studies as demonstrated by (Davidson et al., 2013), (Souverein et al., 2011) and (Franz et al., 2010) for leafy greens in different distribution chains. The behaviour of pathogens on fresh produce is highly investigated by simulating laboratory experiments and modelling (e.g. *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* on leafy greens (Koseki et al., 2012), on fresh-cut iceberg lettuce (McKellar & Delaquis, 2011) or real-life experiments (Seidu et al., 2008; Van der Linden et al., 2013). The refrigerator conditions were also questioned in this survey, with respectively 16% (Belgium) and 15% (Spain) of the respondents having the temperature at 4 °C or lower, 62% (Belgium) and 64% (Spain) between 4 °C and 7 °C, above 7 °C 1% (Belgium) and 4% (Spain) and still 21% (Belgium) and 17% (Spain) of the respondents indicated that they did not know their refrigerator temperature.

Figure 7.2 is illustrating the obtained results for the home washing practices for lettuce head and also pre-packaged lettuce e.g. baby leaf spinach. As could be expected only a minority of the consumers is not washing the lettuce head (respectively 1% in Belgium and 2% in Spain), while 50% of the Belgian respondents and 67% of the Spanish respondents are not washing their pre-packaged lettuce anymore at home. This indicates, surprisingly, that still many consumers are washing again the already industrially washed leafy greens at home. It was recommended that additional washing of ready-to-eat green salads is not likely to enhance safety by an expert group in US (Pangloli et al., 2009). Home washing practices may also influence the final concentration of pathogens on fresh produce and will therefore influence a final risk assessment result as demonstrated by (Barker et al., 2013) or (Pavione et al., 2013).

The effect on washing or even home washing is intensively described in the available scientific literature, but the effect on log reduction of the present bacteria or pathogens is ranging depending on pathogen, commodity, type of washing which is applied (e.g. running tap water or immersion), application of some disinfectants etc. In general with optimum conditions of washing a maximum of 1 up to 2 log reduction can be found, e.g. immersion of strawberries between 0.4 and 0.9 log reduction (Rodgers et al., 2004; Hung et al., 2010) while for leafy greens immersion is reported to range between 0.4 log (Sengun, 2013) and 1.3 log reduction (Beuchat et al., 1998). Rinsing under running tap water is reported to be more effective with reduction rates up to 2.2 logs (Palumbo et al., 2009). However, impact of spin drying or drying in a towel on the removal of pathogens can be debatable.

Table 7.7. Storage time for fresh and packaged vegetables and fruits by consumers in Belgium and Spain.

	Product	Storage Time										
		Lettuce	Fresh Herbs	Strawberry & Raspberry	Berries	Grapes	Apples	Tomatoes	Bell Pepper	Packed Lettuce	Juices and Smoothies	Packed Fruit Salads
Belgium	The same day	157 or 10%	210 or 14%	421 or 27%	378 or 28%	78 or 5%	19 or 1%	28 or 2%	67 or 4%	224 or 19%	210 or 29%	121 or 33%
	1 to 3 days after	1084 or 69%	479 or 32%	959 or 61%	800 or 58%	769 or 49%	163 or 10%	395 or 25%	454 or 30%	745 or 62%	301 or 42%	165 or 44%
	4 to 7 days after	296 or 19%	455 or 30%	184 or 12%	179 or 13%	623 or 40%	692 or 44%	815 or 53%	718 or 48%	171 or 14%	154 or 21%	48 or 13%
	Longer than a week	30 or 2%	341 or 23%	8 or 0%	13 or 1%	81 or 6%	686 or 44%	306 or 20%	259 or 17%	61 or 5%	47 or 7%	35 or 9%
	Longer than expiration date	2 or 0%	17 or 1%	0 or 0%	0 or 0%	0 or 0%	17 or 1%	7 or 0%	7 or 1%	3 or 0%	5 or 1%	4 or 1%
	Number of respondents	1569	1502	1572	1370	1551	1577	1551	1505	1204	717	373
Spain	The same day	25 or 4%	44 or 9%	67 or 12%	70 or 20%	31 or 6%	16 or 3%	16 or 3%	16 or 3%	38 or 7%	85 or 18%	51 or 16%
	1 to 3 days after	233 or 40%	126 or 27%	312 or 54%	157 or 46%	168 or 30%	57 or 10%	101 or 17%	83 or 14%	248 or 49%	125 or 26%	**129 or 42%**
	4 to 7 days after	237 or 40%	161 or 34%	156 or 27%	87 or 25%	242 or 43%	208 or 35%	301 or 50%	252 or 44%	165 or 32%	**137 or 28%**	88 or 29%
	Longer than a week	84 or 14%	124 or 26%	33 or 6%	26 or 8%	109 or 20%	286 or 49%	171 or 28%	209 or 36%	47 or 9%	124 or 26%	35 or 11%
	Longer than expiration date	9 or 2%	20 or 4%	5 or 1%	4 or 1%	8 or 1%	17 or 3%	14 or 2%	15 or 3%	13 or 3%	9 or 2%	5 or 2%
	Number of respondents	588	475	573	344	558	584	603	575	511	480	308

[a] Value listed is the mode

Table 7.8. Storage method for fresh and packaged vegetables and fruits by consumers in Belgium and Spain.

	Product	Storage method										
		Lettuce	Fresh Herbs	Strawberry & Raspberry	Berries	Grapes	Apples	Tomatoes	Bell Pepper	Packed Lettuce	Juices and Smoothies	Packed Fruit Salads
Belgium	On room temperature	23 or 2%	884 or 58%	242 or 15%	248 or 18%	813 or 52%	818 or 51%	382 or 24%	153 or 10%	27 or 2%	15 or 2%	5 or 1%
	In the refrigerator	1477 or 93%	562 or 37%	1273 or 80%	1084 or 78%	684 or 44%	568 or 36%	1075 or 68%	1263 or 82%	**1189 or 98%**	**714 or 97%**	**390 or 97%**
	In the basement	85 or 5%	87 or 5%	80 or 5%	62 or 4%	75 or 4%	203 or 13%	122 or 8%	117 or 8%	2 or 0%	7 or 1%	8 or 2%
	Number of respondents	1585	1533	1595	1394	1572	1589	1579	1533	1218	736	403
Spain	On room temperature	3 or 0%	43 or 9%	16 or 3%	12 or 4%	53 or 10%	157 or 27%	40 or 7%	21 or 4%	3 or 1%	11 or 2%	5 or 2%
	In the refrigerator	572 or 99%	341 or 73%	536 or 95%	316 or 92%	460 or 84%	318 or 55%	526 or 88%	528 or 93%	498 or 98%	449 or 95%	296 or 97%
	In the basement	5 or 1%	86 or 18%	13 or 2%	12 or 4%	37 or 6%	101 or 18%	28 or 5%	18 or 3%	4 or 1%	15 or 3%	3 or 1%
	Number of respondents	580	470	565	340	550	576	594	567	505	475	304

[a]Value listed is the mode

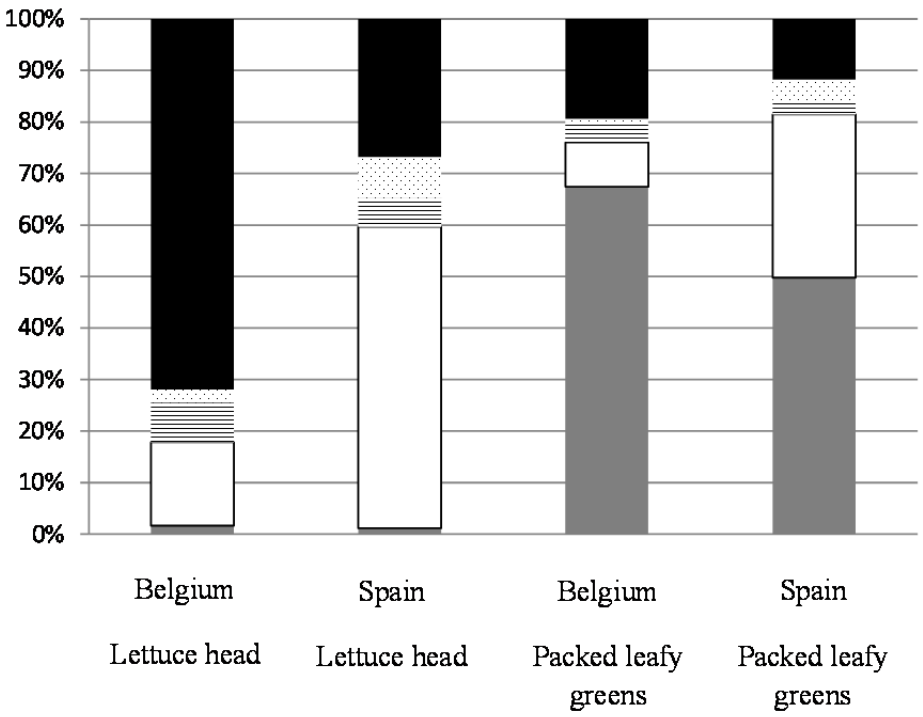

Figure 7.2. Washing procedures of fresh lettuce adopted by the respondents in Belgium and Spain (expressed as % of respondents) for lettuce head and pre-packed leafy greens (black: Washing and drying with a salad spinner; dotted: Washing while scrubbing and drying; stripes: Washing and drying; white: Washing and not drying; grey: no washing)

4. Conclusion

In conclusion, dataset and distribution of consumption of specific fresh produce commodities, often linked towards microbiological or chemical hazards, are available for acute and chronic exposure assessment calculations for Belgian and Spanish consumers, as representatives from Northern and Southern European countries. The Spanish consumption (frequency of consumption and portion size) for target commodities is higher and also the fraction of non-consumers is for most of the commodities lower compared to the Belgian respondents. It is clear that the consumption of raw fruits and vegetables is regionally different. But the ranking of the popular and most consumed commodities is the same in the two countries, being tomato

as vegetable and apple as fruit. The convenience type of products such as fruit mix, vegetables mix and pre-packaged lettuce is clearly more consumed in Spain compared to Belgium. The applied questionnaire is leading to the possibility to calculate the distribution of the daily or usual consumption and the acute consumption for different risk estimation purposes. The survey on consumer handling practices demonstrated that Belgian consumers are having a shorter time period between purchase and consumption, but more often room temperature conditions are applied. While in Spain, all commodities were refrigerated and stored for a longer period. Conditions in the refrigerator were similar in both countries. Investigation of washing practices revealed that the majority of consumers are washing their lettuce head and also many consumers are rewashing the already washed pre-packaged leafy greens at home. The included commodities are currently under further investigation of microbiological and/or chemical exposure assessment calculations and the obtained consumption data and consumer handling practices can be applied for further research.

General Discussion

Extensive research has been performed to demonstrate that foodborne pathogens can contaminate, survive or even growth at various stages of RTE leafy green production chain such as production, harvest, processing, distribution and hand preparation. Although pre-harvest and post-harvest mitigation strategies can reduce the risks, they seem not enough to prevent outbreaks. In Spain, systematic studies describing the current microbial situation and identifying critical points through the production of RTE leafy greens were needed.

Admittedly, there is a wide range of factors affecting the microbial safety of leafy greens at primary production level. Park et al. (2012) carried out a systematic review intended to assess all the previously considered risk factors for contamination of fruit and vegetables with *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157:H7. This study concluded that, based on the existing literature, controlling the contamination of irrigation water and soil were the most promising targets for prevention of produce contamination and stressed the need for confirming the association between risk factors and contamination. In this regard and, specifically in relation to leafy greens, extensive research over the last years has been oriented towards identifying and characterizing the main risk factors affecting microbial safety at field level by means of the study of microbial pathogen prevalence and indicator distribution (Park et al., 2012-2015; Holvoet et al., 2014a,2015; Ceuppens et al., 2015).

On that basis, *Chapters 3* and *4* evaluated the main risk factors affecting the microbial safety of leafy greens at primary production levels. In this sense, the distribution of indicator microorganisms and the prevalence of foodborne pathogens in baby spinach grown in the southeast of Spain were evaluated for the first time. Regarding pathogen prevalence on leafy greens, most of the recent studies have not found either *Salmonella* or pathogenic *E. coli* on leafy greens (Loncarevic et al, 2005;

Bohaychuk et al., 2009; Oliveira et al., 2010; Holvoet et al., 2014a). Our results confirmed that the low prevalence of enteric pathogens on fresh produce makes difficult to find. Consequently, many studies have assessed *E. coli* prevalence as an index organism for the presence of enteric pathogens in leafy greens. Generally, they have reported relatively low values ranging from 5% to 20% in developed countries (Mukherjee et al., 2004; Loncarevic et al., 2005; Bohaychuk et al., 2009; Oliviera et al., 2010; Holvoet et al., 2014a; Park et al. 2013; Cardamone et al., 2015). Our results were in agreement with these data as generic *E. coli* prevalence on baby spinach reported in this PhD was around 5%. Nonetheless, it is important to notice that prevalence data are in many cases difficult to compare due to important differences between studies, such as sampling size, sampling location, climatic conditions and analytical methods. Thus, the absence of positive samples for the presence of pathogens in baby spinach collected during our study could suggest an unrealistic perception of the microbial safety of this commodity since some pressure from environmental samples was noticed, mostly from manure and irrigation water. Therefore, this study confirmed previous research, which highlighted manure as a risk factor for pathogen contamination of leafy greens (Strawn et al., 2013a; Park et al., 2015). As sampling was carried out it conventional farms where composted manure was always applied, our findings suggested failures in the composting process (de Quadros Rodrigues et al., 2014).

In relation to irrigation water, it has been extensively reported as one of the most important risk factors affecting leafy green contamination at primary production level (Pachepsky et al., 2011; Holvoet et al., 2012; Park et al., 2012; Allende & Monaghan, 2015; Strawn et al., 2015; Uyttendaele et al., 2015). However, the most recent data regarding prevalence for *Salmonella* and pathogenic *E. coli* in water have reported relatively low values (de Quadros Rodrigues et al., 2014; Holvoet et al., 2014a; Jones et

al., 2014). *Chapter 3*, and in line with previous findings, confirmed irrigation water as a relevant risk factor for leafy green contamination as *Salmonella* spp. positive samples were found in water samples, although its prevalence was relatively low (2%). Even though the number of tested samples for pathogen presence limited generalizations, the fact that the highest *E. coli* and *Enterococcus* spp. prevalence were found in water samples confirmed irrigation water as a risk factor for leafy green contamination. Laboratory and field studies have shown that pathogens and indicator organisms can be transmitted from irrigation water to produce and they can remain viable for variable periods of time, depending on environmental conditions (Delaquis et al., 2007; Wood et al., 2010; Fonseca et al., 2011). As it has been widely reported in literature, water quality has an important effect on the microbial safety of leafy greens and several strategies have been proposed to reduce the risk of produce contamination with pathogens during irrigation (Pachepsky et al., 2011; Park et al., 2012; Holvoet et al., 2014a; Allende & Monaghan, 2015; Ceuppens et al., 2015; Strawn et al., 2015; Uyttendaele et al., 2015).

Apart from agricultural management practices, it is known that the likelihood of leafy green contamination is strongly related to prevailing weather and climate (Laje et al., 2010; Liu et al., 2013). Several weather factors have been repeatedly reported to have an important impact on the microbial safety (Ivanek et al., 2009; Strawn et al., 2013a; Holvoet et al., 2014; Park et al., 2013-2015). However, the reasons and the inconsistences regarding the relationship between weather factors and microbial safety still remain unclear (Ward et al., 2015). Temperature and precipitation patterns are, for example, closely related with not only the fate and transport of enteric bacteria but also with their survival and growth (Liu et al., 2013). Elevated concentration of microbial indicators or increased pathogen presence have been attributed to several weather

factors such as seasonality (Ailes et al., 2008; Wilkes et al., 2011), warmer temperatures (Brandl & Mandrel, 2002; Holvoet et al., 2014a; Ward et al., 2015), humidity (Brandl & Mandrel, 2002) and greater rainfall (Halley et al., 2009; Setti et al., 2009; Parker et al., 2010; Strawn et al., 2013a). In a recent study by Park et al., (2015), precipitation was a positive predictor of *E. coli* contamination on spinach farms. This study concluded that farm management (i.e. manure application, workers' hygiene practices), environment (i.e. wildlife presence, proximity of animal farms), and weather factors (i.e. temperature and precipitation) jointly influenced the probability of spinach contamination with generic *E. coli*. However, once it had occurred, only weather factors had an effect on *E. coli* counts. In our study, all the positive samples for *E. coli* were found when the temperature was in the highest range (15-20 ºC) which is in concordance with Park et al., (2015) who found a quadratic relationship between generic *E. coli* levels and the maximum daily temperature during the previous days to harvest. It is generally stated that warm seasons are the most critical periods for possible pathogen survival on produce and irrigation water (McEgan et al., 2013). However, as it was mentioned before, the low number of samples made difficult to generalize the findings observed about the relationship between microbial contamination and weather factors. Additionally, the mechanisms underlying the seasonality in foodborne pathogens are not fully understood, but they are likely a complex interplay of different factors (Ward et al., 2015). Moreover, the time frame in which weather factors may affect the microbial safety of fresh produce is not established, since both short and long-term weather potentially contribute to the relationship (Park et al., 2014).

Improving the understanding of these factors is important for better prevention and control of leafy green contamination at pre-harvest level. In this respect, climate change is an important factor to consider as it is expected to strongly affect microbial

safety of fresh produce by changes in precipitations and temperature patterns (Liu et al., 2013) or by increasing the frequency of extreme climatic events (i.e. flooding) (Tirado et al., 2010). Intensive precipitation increases water runoff, which might be an intermediate contamination route of pathogens from manure at livestock farms and from grazing pastures (Parker et al., 2010). In general, it has been established that produce contamination is reduced with longer intervals between flooding and harvest (FDA, 2011). Several studies have attempted to develop recommended intervals between field contamination and harvest but they differ in the establishment of a safety time period (Doyle & Erickson, 2008). Nonetheless, the evaluation of the microbial risk after a flooding is challenging because it is a sporadic event and it is difficult to develop an adequate experimental design. In this line, one of the main achievements of the present PhD thesis was the evaluation of the effects of a flood event on microbial safety of leafy greens, soil and irrigation water in the flood plains after the flooding event. Our findings confirmed flooding as an important risk factor for leafy green contamination and agreed with previous research which suggested the need for mitigation strategies to protect cultivation areas from incidents capable of releasing fecal material (i.e. construction of ditches, establishment of buffer areas). In line with *Chapter 3*, the results obtained in *Chapter 4* highlighted the importance of weather factors after the event. Solar radiation has been reported to be a critical mechanism influencing the survival of microorganisms (Whitman et al., 2004; Sinton 2002, 2007). Accordingly, solar inactivation was probably responsible for the drastic decline observed in pathogens and indicator microorganisms after the flood event.

In general, research carried out to evaluate the likelihood of finding pathogens in primary production and the main risk factors can help to elaborate better recommendations for growers. In our opinion, future research should be oriented

towards the development of appropriated guidelines for pre-harvest application of manure and irrigation water together with improved treatments to guarantee manure safety and water quality. Concerning weather conditions, more research is needed to establish significant relationships between factors and foodborne pathogens. Once established, it might be useful for growers in order to implement preventive measures, which improve safety of leafy greens, such as the establishment of the lag time between a flooding and sowing or harvest. Furthermore, on the basis of season and weather conditions, the identification of risk periods could be possible as it induces a higher state of awareness for growers leading the application of preventive measures (e.g. increased frequency of testing or temporarily use of a water treatment) to avoid microbial contamination of crops.

Recent food contamination events have highlighted the importance of the evaluation of risks associated with the food chain through exposure assessment, a key step in risk assessment. Currently, predictive microbiology (i.e. QMRA and QMEM) is a useful approach to manage food safety risks (Basset et al., 2012).Exposure assessment is objective and evidence-based which leads to more flexibility and enables the elaboration of tailored risk management practices and guidelines (De Keuckelaere et al., 2015). Most of the developed QMRA have focused on enteric pathogens (*Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes*) of leafy greens at different levels: primary production (Franz et al., 2010), from harvest to retail (Koseki & Isobe, 2005), farm-to-consumption (Danyluk & Schaffner, 2011; Ding et al., 2013), factory to consumption (Carrasco et al., 2010) and in specific distribution systems (Pérez-Rodriguez et al., 2011; Tromp et al., 2010). However, and as it has been confirmed in this PhD, enteric pathogens prevalence is assumed to be low and its detection is cost-prohibitive and time-consuming (EFSA, 2014). Furthermore, it has been shown that for pathogens,

performance of sampling is rather poor even when high numbers of samples are tested especially if the sampling is required to be able to detect a low rate of contaminated products (Zwietering et al., 2014). Therefore, generic *E. coli* has been repeatedly used as an index organism for the presence of enteric pathogens (Mukherjee et al., 2007; Park et al., 2013; Holvoet et al., 2014). Thus, no QMEM had been developed on generic *E. coli* on leafy greens at field level. In this PhD collected data of *E. coli* prevalence from *Chapters 3* and *4* together with a literature review of relevant publications was used to develop a QMEM of *E. coli* on baby spinach grown in open-field. In the frame of this PhD, the model was used to evaluate how different scenarios affected *E. coli* loads in different situations, which included weather conditions such as seasonality, rain, solar radiation and simulation of a flooding event as well as different agricultural practices (e.g. different water sources and irrigation methods). Collectively, the results confirmed that both weather factors and management practices influenced the likelihood of *E. coli* contamination in leafy green production environment and that intervention strategies aimed at such factors and practices may reduce the risk of pre-harvest contamination. However, it is clear that 'one-size fit all' approach will not work. As generic *E. coli* may serve as a surrogate organism for enteric pathogens, our findings indicate the potential behaviour of these pathogens under defined situations. This model could be used as a practical and useful approach to evaluate risk factors since simulations could be translatable to other cultivation environments with different weather factors and agricultural practices, leading the risk management in preventing contamination, controlling it and if it occurs, identifying areas that further research or data collection are needed. However, as the outcomes rely partly on assumptions, results should be interpreted as an indication of the level or degree of safety and not as absolute values (De Keuckelaere et al., 2015).

Once the main risk factors at primary production level were established, the next step was taken towards processing level. Several studies aimed to investigate the presence of enteric pathogens in RTE leafy greens have shown that contamination with pathogens occurs infrequently (Koseki et al., 2011; Kokkinos et al., 2012; Pérez-Rodríguez et al., 2014) and even in most of the studies have not found any pathogen in RTE salads (De Giusti et al., 2010; Althaus et al., 2013; Holvoet et al., 2012; Allen et al., 2013; Seow et al., 2013; Jeddi et al., 2014). Our study confirmed that contamination of RTE leafy greens with pathogens is a rare event. This could be explained by the fact that homogenous pathogen contamination of individual units within a batch of fresh produce is not expected (Pérez-Rodríguez et al., 2014). However these findings should be interpreted carefully especially when the number of analyzed samples is relatively low. Nonetheless, part of RTE industry still relies on microbiological testing of end product to evaluate compliance with microbiological standards. Thus, in the leafy green processing industry, there is still a need for the identification of suitable sampling points that improve the detection and increase the amount of produce analysed before distribution.

Several studies have been aimed at assessing the impact of the different processing steps (i.e. washing baths, raw and finished product, surfaces) on the safety of pre-packaged salads (Ailes et al., 2008; Da Cruz et al., 2008; Holvoet et al., 2014). However, none of these studies have found pathogens in the tested samples. Our results were in agreement with previous research as the two positive samples for pathogens were found in water obtained from the centrifuge. This suggests that the origin of the pathogen could be the produce subjected to centrifugation or the process wash water. As mentioned before, *E. coli* has been used as a hygiene or faecal contamination indicator in several studies at processing level (Da Cruz et al., 2008; Althaus et al., 2012; Holvoet

et al., 2012). Holvoet et al. (2012) used this indicator to assess the water management practices in two Belgian fresh-cut companies and found levels up to 5 log CFU/100mL. One of the reasons explaining this *E. coli* accumulation could be that no sanitizer was used to maintain the quality of the water. Therefore, the washing step in the production of RTE salads was identified as a potential pathway for dispersion of microorganisms and introduction of *E. coli* to the end product via cross-contamination. In our study, even though a big number of water samples was analysed, all were negative for generic *E. coli* with the exception of water obtained from centrifugation operation where increasing levels of *E. coli* were found from the beginning until the end of the production day. Our results were in agreement with previous research that highlighted that centrifugation effluent water could be used as a potential sample point to evaluate lot contamination and cross-contamination in the processing chain, even at low levels of pathogen contamination (Tomás-Callejas et al., 2012). In order to gain insight on the relevance of this water as a sampling point, lab scale tests were performed and *E. coli* was recovered in all the effluent water samples but in low numbers, which suggested the need for the filtration of higher volumes of centrifuge water. Therefore, *Chapter 6* provided evidence for the use of centrifuge water as a microbiological quality sampling point in leafy green processing lines. Additionally, it was confirmed that when GMP are strictly applied, pathogenic microorganisms and indicator microorganisms (*E. coli, Enterococcus spp.*) are rarely or even not found in the processing facilities.

Moving to the last step in the farm-to-fork chain, over the last decade several risk assessment studies have been carried out to calculate the exposure of consumers to pathogens through the consumption of fresh produce (Carrasco et al., 2010; Verhoeff et al., 2010; Domenech et al., 2013; Ding et al., 2013; Pielaat et al., 2013; Sant'Ana et al., 2014). As clearly shown, consumer behaviour related to both practices and consumption

patterns, influences exposure and hence risk (CAC, 1999). Therefore, relevant fresh produce consumption data, including frequency and portion size for key populations, are essential for exposure assessment (Donne et al., 2011; Hoelzer et al., 2012). However, relevant national consumption data are not always available for each country and, hence, data derived from other countries and/or populations are frequently used as proxy (Navarro & Jimenez 2011; Barker et al., 2013). Consequently, information about food consumption is essential to estimate exposure of the population to a microbial hazard (Hoelzer et al., 2012). In *Chapter 7*, the suitability of exiting data on the consumption of fresh fruit and vegetables was evaluated for Spain and Belgium. However, for the Spanish data, the portion sizes or units were difficult to identify (Le Donne et al., 2011) and detailed information about actual consumption and the corresponding handling practices were still lacking. This kind of data is necessary in order to develop microbial or chemical exposure assessments linked to fresh produce consumption. Accordingly, this PhD thesis provided standardized data for fresh produce consumed raw or minimal processing that can be used in future exposure assessments related to fresh produce (i.e. leafy greens, pre-packaged salads). Additionally, acute intake was calculated by portion and frequency of consumption of all the commodities. This information could be used in both acute and chronic risk calculations. Regarding handling practices, several QMRA studies have identified produce washing as an main intervention step for lowering enteric disease risks associated with the consumption of fresh produce (Navarro et al., 2009; Ayuso-Gabella et al., 2011; Domenech et al., 2013). With the same purpose, storage duration and temperature have been shown to play an essential role in potential survival, decline, or growth of certain pathogens in RTE products that influence the outcome of microbial risk assessments (Franz et al., 2010; Danyluk & Schaffner, 2011; Pavione et al., 2013). Thus, the standardized data set provided in *Chapter 7* will be very valuable for its use in further research for microbial

exposure assessment linked to leafy greens and acute risk calculations (e.g. risk of *Salmonella* spp. infection from consumption of leafy greens) in Spain and its use should improve the reliability of future microbial risk estimates.

Quantitative microbial risk assessment can provide an objective and scientific basis for risk management decisions. However, the link between risk assessment and risk management is still challenging and further research is needed. Future research should be oriented to the development exposure assessment encompassing all the steps in the Spanish farm-to-fork chain of leafy greens including all the suitable information provided in the present PhD thesis.

Last but not least, one must be aware that despite useful recommendations, GAPs, GMPs and GHPs, it is not possible to obtain zero risk. This cannot be proved through sampling. It is important to understand that a minimally processed produce is a raw ready-to-eat product cultivated in a natural environment especially in open fields. The prevention of contamination is important but there is a continuous pressure of contamination through the whole production chain. Eventually, the communication of the main results achieved in this PhD to growers and processors could be a way of increasing awareness, highlighting the point that presence of enteric pathogens or high levels of indicator are not acceptable. In those cases, mitigation strategies can help to reduce risk associated with microbial contamination.

Conclusions

In the present thesis, it has been shown that in order to avoid microbiological risks associated with leafy vegetables; further work is still needed with the aim of developing good practices focused on reducing identified risk factors. Additionally, at processing plant level, it is also necessary the implementation of good manufacturing practices. The results obtained during this research have shown that microbiological contamination in both, field and processing plant, responds to multiple factors, making it extremely complex to control the microbiological risks.

The main conclusions derived from this thesis are:

1) In relation to climatic factors, it has been shown that temperature and precipitation, exemplified by extreme rain events are critical factors affecting the presence of pathogenic microorganisms as well as counts of indicator organisms in water, soil and leafy green vegetables.

2) The main microbiological risk factors identified as potential sources of pathogenic microorganisms on leafy green vegetables at primary production level are irrigation water and organic fertilizers which were confirmed as main sources of fecal contamination.

3) *E. coli* was confirmed as a possible indicator of fecal contamination and its counts were correlated with higher probability of pathogenic microorganisms.detection.

Taken together, our results at primary production level indicate that a wide range of climatic, agronomic and microbiological factors variables may influence the microbial safety of leafy greens. These factors need to be taken into account in the development of strategies to prevent microbiological contamination of leafy greens.

Data obtained through the systematic study of microbiological hazards during primary production of leafy vegetables represented an optimal dataset for the development of a quantitative exposure assessment of indicators microorganism of fecal contamination (i.e. *E. coli*) in leafy greens at harvest. The results obtained in this thesis show that:

4) Levels of *E. coli* in leafy vegetables at harvest are mainly influenced by seasonality, growing time, irrigation water source and irrigation system used (sprinkler vs. drip irrigation).

Regarding the processing plant IV level, the results obtained in this thesis confirmed that:

5) The implementation of Good Manufacturing Practices along the whole production chain, reduce microbiological risks associated with pathogenic bacteria since no pathogenic microorganisms were detected in any of the sampling points. Additionaly, levels of indicator organisms (*E. coli*) were very low and even below the detection limit, which corroborates the proper functioning of Management Systems Food Security.

In relation to the last step in the food chain, i.e, the consumer, it was demonstrated that there is a lack of date in relation to fresh fruit and vegetables consumption of in different countries of the European Union, including Spain. In relation to this matter this thesis provides:

6) Consumption and handling practices data of fresh fruit and vegetables of Spanish and Belgian consumers. This set of standardized data is valuable and neccesary information that will allow the assessment of the risks associated with microbial exposure after consumption of leafy vegetables in a quantitative microbiological risk analysis.

Conclusión

En el presente trabajo se ha demostrado que para evitar riesgos microbiológicos asociados a las hortalizas de hoja, es necesario seguir trabajando en el desarrollo de buenas prácticas centradas en reducir los factores de riesgos identificados. Asimismo, a nivel de planta de procesado, se deben de implantar las buenas prácticas de fabricación. Los resultados obtenidos a través de esta investigación han demostrado que la contaminación microbiológica en campo y en planta de procesado responde a múltiples factores, lo que hace extremadamente complejo el control de los riesgos microbiológicos. Los factores determinantes definidos en los estudios realizados han sido tipo de producto, las condiciones climáticas, las prácticas agronómicas así como operaciones de procesado, entre otros.

Las principales conclusiones que se derivan de esta tesis son:

1) En relación con los factores climáticos, se ha demostrado que la temperatura y las precipitaciones, ejemplificadas por eventos de lluvia extrema, son factores críticos que afectan a la presencia de microorganismos patógenos así como a los recuentos de microorganismos indicadores en agua, suelo y hortalizas de hoja.
2) Los principales factores de riesgo microbiológico identificados como fuentes potenciales de microrganismos patógenos de hortalizas de hoja a nivel de producción primaria son el agua de riego y los fertilizantes orgánicos fueron confirmados como principales fuentes de contaminación fecal.

3) *E. coli* se confirmó como un posible indicador de contaminación fecal y sus recuentos se correlacionaron con una mayor probabilidad de detección de microorganismos patógenos.

En conjunto, nuestros resultados a nivel de producción primaria indican que existe una amplia gama de variables climáticas, agronómicas y factores microbiológicos que influyen en la seguridad microbiológica y que deben tenerse en cuenta en el desarrollo de estrategias para prevenir los riesgos microbiológicos de los cultivos de hortalizas de hoja.

Los datos obtenidos a través de los estudio sistemáticos de los riesgos microbiológicos durante la producción primaria de hortalizas de hoja, representan una base de datos óptima para realizar una evaluación cuantitativa de la exposición de hortalizas de hoja a microorganismos indicadores de contaminación fecal, como es el caso de *E. coli*, en el momento de la recolección. Los resultados obtenidos en esta Tesis muestran que:

4) Los niveles de *E. coli* en hortalizas de hoja en el momento de la recolección están principalmente influenciados por la estacionalidad, duración del cultivo, el tipo de fuente de agua de riego que se utiliza así como el sistema de riego seleccionado (aspersión vs. goteo).

Con respecto a la planta de procesado de IV Gama, a través de los resultados obtenidos se confirmó:

5) La aplicación de Buenas Prácticas de Fabricación a lo largo de toda la cadena de producción, reduce los riesgos microbiológicos asociados a bacterias patógenas ya que no se detectaron en ninguno de los puntos de muestreo. Asimismo, los niveles de microorganismos indicadores (*E. coli*) fue muy reducido e incluso por debajo de los límites de detección, lo que

corrobora el buen funcionamiento de los Sistemas de Gestión de la Seguridad Alimentaria.

En relación con el último paso en la cadena agroalimentaria, es decir, el consumidor, existe una carencia en cuando a los consumos de frutas y hortalizas en distintos países de la Unión Europea, incluida España. Con respecto a este tema esta tesis aporta:

6) Datos sobre el consumo, la frecuencia y la manipulación por parte del consumidor de frutas y hortalizas frescas en España y Bélgica. Este conjunto de datos normalizado es una información necesaria con el fin de evaluar los riesgos asociados a la exposición microbiana tras el consumo de hortalizas de hoja dentro de un análisis cuantitativo de riesgos microbiológicos.

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Curriculum vitae

Curriculum vitae

Irene Castro Ibáñez was born in Murcia (Spain) on December 8th 1985. After five years, she graduated in 2008 as Biologist at University of Murcia.

In September 2011, she started her PhD research at the Department of Food Science and Technology at CEBAS-CSIC (Murcia, Spain) under the supervision of Prof. Dr. Ana Allende Prieto and Dr. Mª Isabel Gil Muñoz and with the economical support of a JAEPreDoc contract from the CSIC. The research was part of the European FP7 Veg-i-trade project "Impact of Climate Change and Globalisation on Safety of Fresh Produce" (Grant agreement no.: 244994).

During her PhD, she performed research stays in two international laboratories. In 2012 she worked during three months in the Laboratory of Food Microbiology and Food Preservation at the University of Gent under the guidance of Prof. Dr. Mieke Uyttendaele and Prof. Dr. Liesbeth Jacxsens). In 2013, she was working in the Department of Systems Biology at the Technical University of Denmark under the supervision of Prof. Dr. Claus Stenberg. Finally, in 2014 she stayed for two months in the Laboratory of Food Microbiology and Food Preservation at the University of Gent under the same mentioned supervisors.

In the course of her Ph.D, she published as (co-)author in international journals and participated actively in national and international congresses.

UNIVERSITY DEGREE

2003-2008. BSc: Biology, Biosanitary and Biotechnology intensification. University of Murcia (Spain).

2008-2009. Master in Biology and Technology of mammal reproduction. University of Murcia (Spain).

2011-2013. Master in Molecular biology and Biotechnology. University of Murcia (Spain).

PROFESSIONAL BACKGROUND

September 2011-until present: Research Group on Quality, Safety and Bioactivity of Plant Foods, Food Science and Technology Department. Institute of Research CEBAS - CSIC, Murcia (Spain).

February–April 2014. Research stay. Laboratory of Food Microbiology and Food Preservation. Department of Food Safety and Food Quality. University of Gent (Belgium).

August–October 2013. Research stay. Department of Systems Biology. Technical University of Denmark.

April–July 2012. Research stay. Laboratory of Food Microbiology and Food Preservation. Department of Food Safety and Food Quality. University of Gent (Belgium).

RELEVANT SCIENTIFIC PUBLICATIONS

Truchado, P., Giménez-Bastida, J.A., Larrosa, M., **Castro-Ibáñez, I**., Espín, J.C., Garcia-Conesa, M.T. & Allende, A. **2012**. Inhibition of Quorum Sensing (QS) in *Yersinia enterocolitica* by an Orange Extract Rich in Glycosylated Flavanones. *Journal of Agricultural Food Chemistry, 60*, 8885-8894.

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Medina-Martínez, M.S., Truchado, P., **Castro-Ibáñez**, **I** & Allende, A. **2015**. Antimicrobial Activity of Hydroxytyrosol: A Current Controversy. Accepted for publication in: *Bioscience,Biotechnology, & Biochemistry Journal*.

Castro-Ibáñez, **I.**, Jacxsens, L., Gil, M.I., Uyttendaele, M. & Allende, A. Quantitative exposure assessment of *Escherichia coli* in baby spinach production in Spain: Evaluating the effects of interventions and meteorological conditions. Submitted to: *International Journal of Food Microbiology*.

CONGRESSES

Allende, A., Gómez-López, V.M., **Castro-Ibáñez, I.**, Medina, M.S. & Gil, M.I. Agricultural Production and Processing Practices Affecting Safety of Leafy Vegetables. 2ND International CIGR Workshop on Food Safety: Technologies and innovations applied to food safety. 5th – 6th July 2012, Valencia, Spain. Oral Presentaion.

Castro-Ibáñez I., Gil M.I., Tudela J.A. & Allende, A. **2013**. Impact of extreme climatic events on microbial safety of leafy greens: Flooding. International Association of Food Protection. Charlotte, North Carolina. July 28-31 2013. Oral presentation.

Castro-Ibáñez I., Allende, A. & Sternberg, C. **2013**. Inhibition of Biofilm Formation in Salmonella spp. by Chlorine Simulating Dynamic Conditions in the Washing Tank of Fresh Produce.) 1st Conference of the COST Action: A European Network for Mitigating Bacterial Colonization and Persistence on Foods and Food Processing Environments. Prague (Czech Republic). November 27-28, 2013. Poster.

Castro-Ibáñez I., Gil, M.I. & Allende, A. **2014**. Lessons Learnt from Systematic Sampling in Leafy Greens Production Chain. Consortium meeting of European Proyect Veg-i-Trade. University of Pretoria (Southafrica). March 17-24, 2014. Oral presentation.

Castro-Ibáñez I., De Keuckelaere, A., Delbeke, S., Uyttendaele, M. & Jacxsens, L. **2014**. Quantitative exposure/risk assessment on fresh produce for enteric bacteria. Consortium meeting of European Proyect Veg-i-Trade. University of Pretoria (Southafrica). March 17-24, 2014. Oral presentation

Castro-Ibáñez I., Jacxsens, J., Kirezieva, K., Luning, P.A., Gil, M.A., Uyttendaele, M. & Allende, A. **2015**. Maturity of Food Safety Management System

(FSMS) along the Fresh Produce Chain in Spain. Innovation in Integrated & Organic Horticulture. International Symposium. Universidad de Avignon (Francia). Junio 08-12, 2015. Presentación Oral.

POSTGRADUATE COURSES

2012. Gene regulation and next generation sequencing. Consolider Proyect. CEBAS-CSIC. Spain. Duration: 30 hours.

2013. Microbial Risk Assesment: from Science to the Food Industry. University of Córdoba. Spain. Duration: 8 hours.

2013. Ph.D course in Biofilm Techniques for Food Research. University of Copenanghen and Technical University of Denmark. Duration: 50 hours

2014. Food Safety. Old pathogens, new challenges. Asociación de Veterinarios Municipales. Murcia (Spain). Duration: 5 hours

2014. Scopus – Database. Fundación Española para la Ciencia y la Tecnología. Murcia (Spain). Duration: 4 hours

LANGUAGES

2010. First Certificate of English (B2). University of Cambridgue.

2015. Certificate in Advanced English (C1). University of Cambridgue.