Is your salad safe to eat?

Aspects of foodborne zoonotic bacteria in ready-to-eat leafy vegetables and mixed-ingredient salads

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Abstract

Ready-to-eat (RTE) leafy vegetables or mixed-ingredient salads are a popular part of the modern healthy diet. Contamination of these products with bacterial pathogens can occur during any step in the production chain and, since there is no step that kills pathogens during the production of RTE salads, a completely safe final product can never be guaranteed.

In fact, almost 10% of RTE mixed-ingredient salads from Swedish retail outlets tested in this thesis were contaminated with foodborne pathogens or presumptive pathogens. *Listeria monocytogenes* was isolated from two out of 141 samples analysed. The other findings included detection of virulence genes present in pathogenic *Yersinia enterocolitica* and shiga toxin-producing *Escherichia coli* (STEC), but these could not be culturally confirmed.

In growth trials, it was found that mixing RTE baby spinach with cooked chicken (representing a mixed-ingredient salad) strongly influenced growth of inoculated *L. monocytogenes*, pathogenic *Y. enterocolitica* and *E. coli* O157:H7 *gfp*+ during storage under temperature abuse (15 °C). Mixed-ingredient salad also supported growth of *L. monocytogenes* under storage conditions recommended for this product in Sweden (8 °C for three days). The estimated risk of listeriosis was 16-fold higher on consuming a mixed-ingredient salad stored at 8 °C at the end of shelf-life, or 200 000-fold higher when the salad was stored at 15 °C, compared with consumption on the day of inoculation. Hence, preventing temperature abuse during storage is of critical importance in mitigating the risk of foodborne listeriosis from these mixed-ingredient salads.

The microbiota of RTE baby spinach and mixed-ingredient salad during the growth trials was studied by Illumina 16S rRNA amplicon sequencing. This molecular method revealed changes in the bacterial communities during storage at 8 or 15 °C and correlations were observed between viable counts of inoculated strains and abundances of some taxonomic orders. However, this method was not useful in identifying human pathogens in the salads, even when these were present in high numbers that can cause disease in humans.

Keywords: baby spinach, food safety, growth potential, *Listeria monocytogenes*, microbial contamination, microbiota, pathogenic *Yersinia enterocolitica*, Shiga toxin-producing *Escherichia coli,* temperature abuse

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Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Söderqvist K**, Thisted Lambertz S, Vågsholm I, Boqvist S (2016). Foodborne bacterial pathogens in retail prepacked ready-to-eat mixed ingredient salads. *Journal of Food Protection* 79(6), 978-85.

II **Söderqvist K**, Thisted Lambertz S, Vågsholm I, Fernström L-L, Alsanius B, Mogren L, Boqvist S (2017). Fate of *Listeria monocytogenes*, pathogenic *Yersinia enterocolitica* and *Escherichia coli* O157:H7 *gfp*+ in ready-to-eat salad during cold storage: What is the risk to consumers? *Journal of Food Protection* 80(2), In press.

III **Söderqvist K***, Ahmed Osman O*, Wolff C, Bertilsson S, Vågsholm I, Boqvist S. Emerging microbiota during cold storage and temperature abuse of ready-to-eat salad. Submitted manuscript. (*joint first authors)

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The contribution of Karin Söderqvist to the papers included in this thesis was as follows:

I Determined sampling plan and laboratory analyses in cooperation with the co-authors. Performed sampling and analyses. Drafted the manuscript and handled correspondence with the journal.

II Mainly responsible for laboratory experiment design and performed the majority of the labwork. Mainly responsible for data interpretation in collaboration with the co-authors. Drafted the manuscript and handled correspondence with the journal.

III Collected the samples. Mainly responsible for data interpretation in collaboration with the co-authors. Drafted the manuscript and handled correspondence with the journal.

Abbreviations

16S rRNA	16S ribosomal RNA
CAY	CHROMagar Yersinia
CFU	Colony-forming units
CIN	Cefsulodin-irgasan novobiocin
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *Escherichia coli* (STEC strains capable of causing infection in humans)
EU	European Union
EURL	European Union Reference Laboratory
FAO	Food and Agriculture Organization of the United Nations
FDA	U.S. Food and Drug Administration
Gfp	Green fluorescent protein
GMP	Good manufacturing practice
ISO	International Organization for Standardization
NFA	Swedish National Food Agency
NMKL	Nordisk Metodikkommitté för Livsmedel
PCR	Polymerase chain reaction
RTE	Ready-to-eat
SLU	Swedish University of Agricultural Sciences
spp.	Species
STEC	Shiga toxin-producing *Escherichia coli* (may also be referred to as verocytotoxin-producing *E. coli* (VTEC))
U.K.	United Kingdom
U.S.	United States
WHO	World Health Organization

1 Introduction

Healthy and convenient foods such as ready-to-eat (RTE) leafy vegetables or mixed-ingredient salads have become popular in many countries in recent years (Ragaert *et al.*, 2007; EC, 2005a). During the same period, there has been an increase in the number of foodborne illnesses associated with vegetables (EFSA, 2013b; WHO & FAO, 2008). Leafy vegetables are normally not subjected to any step that eliminates pathogens before consumption (*e.g.* heat treatment) and thus, while being important for human health, they must also be considered potential health risks.

1.1 Why eat fruit and vegetables?

Fruit and vegetables are often referred to as one single food group, including berries but usually not potatoes. Apart from being rich sources of vitamins, minerals and fibre, fruit and vegetables also contain a wide variety of phytochemicals (e.g. carotenoids and flavonoids) that have been reported to prevent some of the processes involved in the development of cancer and cardiovascular disease (Rodriguez-Casado, 2016). Increased consumption of fruit and vegetables and decreased consumption of meat or carbohydrate-rich foods (*e.g.* pasta, bread) also prevents obesity (WHO, 2003b).

In a world threatened by global warming and scarcity of natural resources, there is a call for environmentally sustainable diets. Adopting a diet with more plant-based foods and less meat would reduce greenhouse gas emissions and ease the pressures on scarce drinking water supplies (Water Footprint Network, 2016; McMichael *et al.*, 2007).

Since fruit and vegetables are important components of a healthy and environmentally friendly diet, and sufficient daily consumption of these could help prevent major diseases, a minimum intake of 400 g fruit and vegetables per day is recommended by the World Health Organization (WHO, 2003b).

However, most populations world-wide are not consuming nearly enough fruit and vegetables (WHO, 2003a). The Swedish National Food Agency (NFA) recommends a daily fruit and vegetable intake of at least 500 g (Livsmedelsverket, 2016b) but, although the consumption of vegetables has almost tripled over the past 50 years, the average Swede still consumes less than 400 g per day (Jordbruksverket, 2014).

In recent years, the relationships between diet, gut microbiota and health have been intensively studied and, although certain compositions of microbiota appear to be beneficial to host health, the mechanisms are far from clear (Salonen & de Vos, 2014). However, the composition of the diet shapes the gut microbiota by providing a selective growth advantage for certain bacteria (Salonen & de Vos, 2014). For example, it has been suggested that some components of leafy vegetables have beneficial effects on human gut microbiota (Speciale *et al.*, 2016; Montelius *et al.*, 2013). It has also been suggested that the microbiota of plants *per se* has a positive effect on human health by stimulating the immune system and enhancing microbial diversity in the gut microbiome (Berg *et al.*, 2014).

1.2 Trends in leafy vegetable consumption

During recent decades, there have been changes in the production of leafy vegetables and consumer habits that may have food safety implications. A greater diversity of fresh vegetables than ever is now available to consumers in many high-income countries, regardless of season. In countries such as Sweden with a cool climate, the supply is dependent on import of fresh vegetables from other parts of the world (Little & Gillespie, 2008) in which sanitary conditions may be poor during growth and processing (Kirezieva *et al.*, 2015).

As part of the trend for eating fresh and raw food, vegetables that have traditionally been cooked, such as spinach and kale, are increasingly being consumed raw. In addition, products that are prepared and ready-to-eat (RTE), for example bags containing leafy vegetables that have already been shredded and washed, are becoming increasingly popular (Figure 1). Ready-to-eat foods depend on refrigeration as the primary means of preservation (EC, 2005b). They do not need to be cooked or processed (*e.g.* washed) to eliminate microorganisms or reduce them to an acceptable level before consumption (EC, 2005b). The availability of bagged salads and other RTE vegetables has increased exponentially since their introduction on the United States (U.S.) market in the early 1980s (Garrett *et al.*, 2003). In Sweden, these products were introduced many years later, in 2005, when one of the largest supermarket chains (ICA) started to sell RTE leafy vegetables, with approximately 600 000 bags sold in

that year. In 2016, the number of bags sold per year had increased to almost 40 million (personal communication, ICA).

Figure 1. Bags of ready-to-eat (RTE) leafy vegetables at retail in Sweden with "washed & ready to use" indicated on the packages. One of the bags have a sticker that says "Buy now – eat soon! 50% off. Close to best-before date. Save the environment and money" (photo by author).

To supply consumer demand, production of leafy vegetables such as lettuce and spinach has increased consistently since the early 1990s (FAO, 2016). In addition, the production and processing steps have been intensified and centralised, with large volumes of leafy vegetables being prepared at packing facilities and distributed over large areas, sometimes including several countries (Lake *et al.*, 2005; Tauxe *et al.*, 1997).

With changing food production practices, storage conditions and eating habits, new pathogen-commodity combinations presenting food safety risks have emerged (Beuchat & Ryu, 1997). These include *e.g.* shiga toxin-producing *Escherichia coli* (STEC) and leafy vegetables (Tauxe *et al.*, 1997). There have also been demographic changes, with an increasing number of elderly and immunocompromised consumers, who are more susceptible to foodborne disease, within the population in high income countries (Lake *et al.*, 2005; Tauxe *et al.*, 1997).

1.3 Definition of leafy vegetables and mixed-ingredient salads

Leafy vegetables include all vegetables of a leafy nature and of which the leaves (and core) are intended to be consumed raw (WHO & FAO, 2008). 'Leafy greens' is a term that is also used for these vegetables, but it may be misleading since the leaves of some varieties may not be green (WHO & FAO, 2008), *e.g.* radicchio and lollo rosso have red leaves. Baby leaves, *e.g.* baby spinach and rocket, are leafy vegetables harvested at a young age. Leafy vegetables have high

water activity and, while the nutrient levels of protein and carbohydrates are low (1-3%) (Roe *et al.*, 2013), they represent a supportive substrate for microorganisms (Franzetti *et al.*, 2015).

In recent years, there has been a development whereby a diversity of ingredients are added to vegetables to produce a wide range of salad meals for consumers (Little & Gillespie, 2008). Different terms have been used to describe this food product. 'Pre-packaged mixed vegetable salads' has been used by some (Little *et al.*, 2007; Anonymous, 2005), while 'mixed-ingredient salads' has been used by others (Bovo *et al.*, 2015). This thesis uses the term 'mixed-ingredient salad', defined as a RTE product containing ingredients of both animal and non-animal origin (Figure 2). These may be combinations of raw (*e.g.* leafy vegetables and tomatoes) and processed (*e.g.* chicken, salmon, ham, pasta and couscous) ingredients. However, when a high-protein food, *e.g.* cooked meat, is added to leafy vegetables, the final product represents a better substrate for bacterial growth, since denatured protein has high concentrations of accessible nutrients, neutral pH and high water activity (Jay *et al.*, 2005).

Figure 2. A commercial mixed-ingredient salad containing leafy vegetables, tomatoes, chicken, bacon and cheese (photo by author).

The mixed-ingredient salads referred to in this thesis are those prepared by a manufacturer or in-store, packaged in plastic bowls and intended as whole meals Figure 2). These mixed-ingredient salads are often readily available in the chill

cabinets of supermarkets, cafés and convenience stores and may be stored for a few days before consumption. A salad dressing is sometimes provided, but in a separate package intended to be added at the time of consumption, in order to avoid impairing the fresh appearance and crispy texture of raw vegetables (Nguyen-The *et al.*, 1996). Mixed-ingredient salads that are prepared in salad bars based on customer preferences, and usually consumed instantly, are not addressed in this thesis. The term 'deli salads' may be synonymous with 'mixed-ingredient salads' but may also refer to salads based on mayonnaise, which are not considered in this thesis.

1.4 Microbiota of leafy vegetables

The microbiota is the community of microorganisms present in an environmental habitat (Marchesi *et al.*, 2016). The leaf surface (the phyllosphere) is a habitat with a diverse mixture of bacteria, yeasts and fungi (Abadias *et al.*, 2008) that may have commensal, pathogenic and mutualistic interactions with the plant host. Bacteria are the most abundant colonist, with densities reaching 10^8 cells per cm^2 (Lindow & Brandl, 2003). The leaf surface of leafy vegetables during cultivation in the field is considered to be a hostile environment for bacteria, due to rapid fluctuations in UV radiation, temperature and humidity and inconsistent availability of nutrients (Lindow & Brandl, 2003). Bacteria are therefore often present in aggregates of different species embedded in microbial polysaccharides in protected areas of the leaf surface (Monier & Lindow, 2004).

Each vegetable has its own microbiota, the composition of which is influenced by field conditions and the series of processing steps, including handling, contact with equipment, washing, packaging and storage, that the leafy vegetable undergoes (Caldera & Franzetti, 2014; Francis *et al.*, 1999). Many of the indigenous bacteria on leafy vegetables are Gram-negative, including members of the *Pseudomonadaceae* and *Enterobacteriaceae* families (Rastogi *et al.*, 2012; Rudi *et al.*, 2002). Species of these families are widely distributed in the environment and several of the species are potential plant pathogens or strains that cause spoilage during storage of leafy vegetables (Jay *et al.*, 2005). Bacteria may also be found within plants as endophytic bacteria originating from soils surrounding the plant roots or from leaf surfaces that then enter the host through the root system or via stomata or wounds because of mechanical damage (Jackson *et al.*, 2013). Food microbiological analysis to date has generally been based on culture-dependent approaches, with molecular methods only recently being introduced (Ceuppens *et al.*, 2014). Molecular DNA-based methods enable detection of a broader range of bacteria that may have escaped detection

by conventional cultivation and are now being applied to investigate the composition of food microbiota.

Most microorganisms associated with leafy vegetables are considered harmless to humans, but some may be opportunistic human pathogens (Berg *et al.*, 2014). When human pathogens occasionally contaminate plant surfaces (see section 1.6), they must co-exist with a diverse community of bacteria that are adapted to phyllosphere conditions (Brandl, 2006). Thus the fate of pathogens is determined by their ability to compete with these bacteria (Cooley *et al.*, 2006). Pathogens in their animal or human host are not exposed to the extreme environmental fluctuations that they experience on the leaf surface. However, biofilms on the leaf may be a protective environment for pathogens. Alternatively, they may convert to a non-culturable state that allows them to survive under harsh conditions (Melotto *et al.*, 2014). Washing steps merely reduce the microbial load by 90-99% (Beuchat & Ryu, 1997; see also section 1.6.2) and are unable to eliminate potential internalised pathogens (Hou *et al.*, 2013).

During processing, *e.g.* washing or cutting, the natural protective barriers of leafy vegetable cells are damaged and the intracellular nutrients they release may further enhance bacterial growth (Brackett, 1994). Studies of some bacterial human pathogens have shown that they preferentially attach to favourable microsites such as cut surfaces (Takeuchi *et al.*, 2000) and that damage to leaves promotes significant bacterial multiplication (Koukkidis *et al.*, 2017; Brandl, 2008). Storage conditions may also further influence the resident microflora (Lopez-Velasco *et al.*, 2011). The final product that is consumed contains a large number of viable microorganisms, ranging from 10^7 to 10^9 viable cells per gram (Nousiainen *et al.*, 2016; Badosa *et al.*, 2008), while occasionally also featuring foodborne pathogens.

The native microbiota present on the surface of fresh produce can play an important role, as its members compete with pathogens for physical space and nutrients and/or produce antagonistic compounds that negatively affect the viability of pathogens (Liao & Fett, 2001). Some bacterial strains isolated from leafy vegetables, including isolates of *Pseudomonas* spp., *Enterobacter* spp. and *Pantoea* spp., have been shown to have an inhibitory effect against a range of pathogens under laboratory conditions (Oliveira *et al.*, 2015a; Schuenzel & Harrison, 2002). It has been suggested that intentional addition of such antagonistic strains, that are able to cope with the cool storage conditions typically used for commercial RTE salad products, could serve as biocontrol agents to reduce the risk of pathogens colonising leafy vegetables (Oliveira *et al.*, 2015a; Critzer & Doyle, 2010), but the efficiency of such procedures has yet to be demonstrated.

1.5 Foodborne pathogens associated with leafy vegetables or mixed-ingredient salads

1.5.1 Leafy vegetables as sources of foodborne disease outbreaks

To date, foods of animal origin have been the main source of documented and reported outbreaks of foodborne disease and 90% of the outbreaks in the European Union (EU) are still associated with foods of animal origin. However, the number of outbreaks and human cases associated with food of non-animal origin appears to be increasing (EFSA, 2013b). In a risk assessment by the Food and Agriculture Organization of the United Nations (FAO), leafy vegetables were ranked as the highest priority in terms of the safety of fresh fruit and vegetables (WHO & FAO, 2008). In a scientific opinion on the risk posed by pathogens in food of non-animal origin based on different criteria, leafy vegetables and *Salmonella* spp. or norovirus were among the top-ranked pathogen-commodity combinations (EFSA, 2013b). An increasing number of outbreaks and illnesses associated with leafy vegetables has also been observed in other parts of the world (Herman *et al.*, 2015). In the U.S., between 1973 and 2012 norovirus was associated with half of reported leafy vegetable-related disease outbreaks with confirmed aetiology, followed by STEC (18%) and Salmonella (11%). A majority of these U.S. outbreaks were attributed to leafy vegetables that had been prepared in a restaurant or catering facility, highlighting the importance of hygienic handling practices by food workers (Herman *et al.*, 2015).

The increased number of foodborne disease outbreaks associated with leafy vegetables reflects to some extent recent improvements in surveillance and investigation of foodborne disease outbreaks, for example improved systems for reporting (Herman *et al.*, 2015). In addition, there have been improvements in laboratory diagnostics for some pathogens, for example norovirus (Widdowson *et al.*, 2005). However, confirming leafy vegetables as the source during investigations of foodborne disease outbreaks is still very challenging. By the time the outbreak is confirmed, the contaminated leafy vegetables have usually already been consumed or discarded, with nothing left to analyse. In addition, leafy vegetables are often consumed with other foods, in a composite food or as a garnish (Herman *et al.*, 2015). Identifying the contaminated ingredient in such dishes can be difficult, requiring detailed information on the constituent ingredients (Herman *et al.*, 2015). Consequently, in most outbreaks associated with leafy vegetables, the association is based on evidence from epidemiological studies.

In Sweden, probably the most well-known outbreak is an *E. coli* O157:H7 outbreak in 2005 that was epidemiologically associated with iceberg lettuce, with 135 reported cases of illness (Soderstrom *et al.*, 2008). The STEC strains isolated from the cases and in cattle at a farm upstream from the irrigation point for the lettuce crop were found to be identical (Soderstrom *et al.*, 2008). Another well-known outbreak world-wide is the *E. coli* O157:H7 outbreak associated with RTE spinach in the U.S. in 2006. It caused 205 confirmed cases of illness and three deaths (FDA, 2007). Despite major efforts, the source of contamination was never determined, but presence of wild boars/feral pigs in the field and proximity of wells used for irrigation water to cattle and wildlife faeces were suspected (California Department of Health Services & FDA, 2007). Apart from the direct effects on human health, enormous economic losses have been incurred by outbreaks or recalls of contaminated leafy vegetables (Melotto *et al.*, 2014). The U.S. outbreak in 2006 caused suspicion among the general public about consumption of spinach. The food safety of RTE leafy vegetables had gained little attention before that outbreak and it actually served as a catalyst for research efforts to enhance the safety of RTE leafy vegetables (Hirneisen *et al.*, 2012). Examples of other, more recent, outbreaks associated with leafy vegetables are presented in Table 1.

Figure 3. A ready-to-eat (RTE) salad mix with leafy vegetables originating from three different countries: Holland, Italy and Sweden (photo by author).

Table 1. *Examples of foodborne disease outbreaks linked to leafy vegetables during the past 10 years, starting with the most recent*

Year	Country	Product	Pathogen	Cases (deaths)	Reference
2016	U.S.	RTE salad mix	*L. monocytogenes*	19 (1)	www.cdc.gov
2016	U.K.	Salad mix	*E. coli* O157	161 (2)	www.gov.uk
2015	U.K.	Pre-packed salad	*E. coli* O157	40	www.gov.uk
2014	Norway	Salad mix	*Y. enterocolitica* O:9	133	(MacDonald *et al.*, 2016)
2012	U.S.	Pre-packed organic spinach and salad mix	*E. coli* O157	33	www.cdc.gov
2011	Norway	Salad mix	*Y. enterocolitica* O:9	21	(MacDonald *et al.*, 2011)
2011	U.S.	Romaine lettuce	*E. coli* O157	58	www.cdc.gov
2010	U.S.	Romaine lettuce (shredded)	*E. coli* O145	26	www.cdc.gov
2010	Denmark	Lollo bionda lettuce (from France)	*Norovirus*	412	www.ssi.dk
2008	Finland	RTE iceberg lettuce	*Salmonella* Newport/Reading	107	(Lienemann *et al.*, 2011)
2007	Sweden	Baby spinach (imported)	*S.* Paratyphi B variant Java *(Salmonella* Java*)*	172	(Denny *et al.*, 2007)

The fact that many of the leafy vegetable products implicated in foodborne disease outbreaks are mixes of varieties with different origins further complicates tracing back possible sources of contamination (Figure 3). One recent example illustrating this is an outbreak of yersiniosis in Norway in 2011 (MacDonald *et al.*, 2011), also presented in Table 1. The product implicated was a salad mix that contained four different varieties of leafy vegetables, originating from 12 suppliers in two countries. The vegetables were mixed, washed in potable water and packaged in Norway. The source of contamination was not identified, but after voluntary withdrawal of the salad mixes by the Norwegian company there were no more reported cases (MacDonald *et al.*, 2011).

Leafy vegetables are usually important ingredients in foods such as mixed-ingredient salads and sandwiches. In 2010, there were 20 different foodborne disease outbreaks in Denmark associated with one batch of lettuce grown in France, which had primarily been used in sandwiches prepared by catering companies. Both norovirus and enterotoxigenic *E. coli* (ETEC) were detected in

food samples. This one incident accounted for about one-quarter of all registered foodborne outbreaks in Denmark in 2010 (Ethelberg *et al.*, 2010).

1.5.2 Human pathogens in leafy vegetables

There are many foodborne pathogens that are relevant for RTE leafy vegetables, including bacteria, viruses and parasites. Among the viruses, not only norovirus but also Hepatitis A has been linked to leafy vegetables. *Cyclospora* spp., *Cryptosporidium* spp., *Giardia* spp. and *Toxoplasma gondii* are examples of protozoan parasites that have been associated with foodborne illness from consuming leafy vegetables. Some important bacterial pathogens that have been associated with leafy vegetables are *Salmonella* spp., STEC, *Campylobacter* spp., pathogenic *Y. enterocolitica* and *L. monocytogenes* (Gorni *et al.*, 2015; EFSA, 2013b), which are briefly summarised in Table 2.

The prevalences of human pathogens have generally been reported to be low in studies examining the microbiological quality of RTE leafy vegetables, as illustrated in Table 3. However, the analytical methods used in these studies are not consistent and in some cases it is not clear whether the leafy vegetables analysed are RTE or not, and hence results may be difficult to compare (WHO & FAO, 2008). However, despite contamination being rare, some of the contaminants involved can cause disease even when present in very low numbers. Since very large quantities of leafy vegetables are consumed and most are consumed raw, even minor contamination of salads can pose potential risks for public health.

Table 2. *Summary of common foodborne pathogens* (*Source: Granum, 2015 and the following websites:* sva.se, slv.se, folkhalsomyndigheten.se, cdc.gov, vetbact.org). [1]A case resulting from exposure to the pathogen outside Sweden. Indicated as mean percentage during 2011-2015

	Salmonella spp.	STEC	*Campylobacter* spp.	*Pathogenic Yersinia enterocolitica*	*Listeria monocytogenes*
Basic facts	G- rod, facultatively anaerobic. Growth >7-8 °C	G- rod, facultatively anaerobic, acid resistant. Growth >7-8 °C	G- spiral-shaped rod, microaerophilic, sensitive to freezing/ drying. Growth 30-45 °C	G- rod, facultatively anaerobic. Growth >-1 °C	G+ rod, facultatively anaerobic, resistant to acid and high salt. Growth >2 °C
Important species/serotypes	Approx. 2300 serotypes, *e.g. S.* Enteritidis and *S.* Typhimurium	O157 most important, O26, O45, O103, O111, O121, O145	*C. jejuni*, *C. coli*	O:3, O:9	
Reservoir	Faeces of animals	Faeces of ruminants (cattle important)	Faeces of animals (poultry important)	Faeces of animals (pigs important)	Environment
Exposure level that has been linked to clinical disease	100 000	<100, no need to grow in food	<1000, no need to grow in food	10^6-10^7	10^7-10^8
Symptoms (sequelae)	Diarrhoea, fever, abdominal pain (reactive arthritis)	Bloody diarrhoea, abdominal pain (haemolytic uremic syndrome)	Diarrhoea, abdominal pain (reactive arthritis, Guillain-Barré syndrome)	Diarrhoea, abdominal pain, fever (reactive arthritis)	Septicaemia, meningitis, stillbirth, premature delivery
Risk groups		Children <5 years, elderly		Children <5 years	Elderly, pregnant women, immuno-compromised individuals
Reported cases/year in Sweden, 2011-2015 (imported cases[1]), trend	2211 to 2917 (74%), ↘	472 to 551 (45%), ↗	7899 to 9179 (52%), ↗	245 to 350 (21%), ↘	56-125 (4%), ↗

Table 3. *Prevalence of human pathogens and Escherichia coli as a hygiene indicator organism in studies of leafy vegetables*

Product	Origin	Number of samples, year of investigation	*E. coli* O157	*Salmonella* spp.	*Campylobacter* spp.	*L. monocytogenes*	Pathogenic *Y. enterocolitica*	*E. coli*	Reference
Bagged leafy vegetables	Retail, Finland	n=100, 2013	7% *stx*-positive	2% PCR-positive	-	2%	2% *ail*-positive	15%	(Nousiainen *et al.*, 2016)
RTE leafy vegetables	Retail, New Zealand	n=307, 2012	0%	0%	0%	0%	-	3.8%	(Hewitt & Rivas, 2015)
RTE lettuce	Retail, Switzerland	n=142, 2011	0.7% *stx*-positive	0%	-	3.5%	0%	3.5%	(Althaus *et al.*, 2012)
Leafy vegetables	Wholesalers, Denmark	n=105, 2009-2010	-	0%	2.9%	-	-	1% (>100 CFU/g)	(Anonymous, 2011)
RTE leafy vegetables	From producers, Italy	n=699, 2005-2007	0%	0%	-	0.3% PCR-positive	-	37% (>100 CFU/g 1st day after packing)	(De Giusti *et al.*, 2010)
RTE vegetables	Retail, Spain	n=236, 2005-2006	0%	1.7%	0%	0.8%	0%	11.4%	(Abadias *et al.*, 2008)
RTE vegetables	Retail, UK	n=3852, 2001	0%	0.1%	0%	2.3%	-	1.3% (0.5% >100 CFU/g)	(Sagoo *et al.*, 2003b)
Open RTE salad vegetables	Catering, retail etc., UK	n=2950, 2001	0%	0%	0%	3%	-	7% (3% >100 CFU/g)	(Sagoo *et al.*, 2003a)

1.5.3 Human pathogens in mixed-ingredient salads

Foods with leafy vegetables as one of several ingredients, such as mixed-ingredient salads, may be contaminated not only by the leafy vegetables but also by the other ingredients. These ingredients may be raw or processed. For example, meat ingredients may be contaminated during slaughter or in subsequent processing steps. Meat ingredients, for example poultry meat and ham, are generally heat-treated before preparation, but may be contaminated after this treatment, *e.g.* when diced or sliced. Improper heating or cooling of meat or carbohydrate ingredients may jeopardise the microbiological safety of these products. When several ingredients are mixed together, cross-contamination may occur. Preparation of mixed-ingredient salads requires human handling, which presents an additional risk of bacterial contamination. Sub-functional cold storage for the final product may also play an important role in determining the microbiological quality of the final mixed-ingredient salad product. There is little information about outbreaks of foodborne pathogens associated with RTE mixed-ingredient salads, but there have been recalls because of findings of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* in internal monitoring of these products by food companies (Food Poisoning Bulletin, 2015; LIDL, 2015; Svenska Dagbladet, 2012).

Because of the increased consumption of RTE food, such as mixed-ingredient salads, and the risk they may pose to public health, in 2005 the European Commission (EC) launched a programme to assess the bacteriological safety of mixed-ingredient salads at retail, with presence of *L. monocytogenes* as the primary concern (EC, 2005a). This resulted in official reports from Ireland and the United Kingdom (U.K) of *L. monocytogenes* contamination in 2.7% (130/2686) and 4.8% (19/714), respectively, of mixed-ingredient salads analysed (Little *et al.*, 2007; Anonymous, 2005). The Irish salad samples were also analysed for *Salmonella*, but this pathogen was not detected. With the exception of these official reports, to my knowledge there have been few studies investigating the microbiological quality of mixed-ingredient salads. However, results from a small Turkish study showed that *L. monocytogenes* was present in 13% of 52 sampled Caesar salads, containing lettuce and chicken, while *Salmonella* was present in 12% (Gurler *et al.*, 2015).

1.6 Contamination from farm to fork

Contamination of leafy vegetables with bacterial pathogens can occur during any step in the production chain. The microbiota of leafy vegetables may be altered by various inputs in the field environment, such as water used for irrigation, animal manure used for fertiliser, livestock in the proximity of fields or access of wild life to the fields (Herman *et al.*, 2015; EFSA, 2013b). Other possible sources of contamination include field workers with poor hygiene and equipment used during harvest and transport to the processing plant (Beuchat & Ryu, 1997). Postharvest contamination may occur via cross-contamination during washing or poor hygiene practices. There is no step that kills any pathogen possibly present during the production and processing of RTE leafy vegetables and thus a completely safe final product may never be guaranteed. However, good hygiene practices during production and processing, a washing step and a controlled cold chain can help to produce a product with high microbiological standard and acceptable safety.

1.6.1 Pre-harvest contamination

Field environment

Contamination of leafy vegetables can never be completely controlled in an environment where wild animals, birds, insects and grazing ruminants are natural inhabitants. Cattle are considered to be the main reservoir for *E.coli* O157:H7 and infected cattle shed the bacteria in faeces without showing clinical signs of disease (Hancock *et al.*, 2001). Other ruminants may also be reservoirs, for example sheep (Soderlund *et al.*, 2012) and deer (Laidler *et al.*, 2013). Other animals may also be important as transient hosts for STEC, for example feral pigs and wild boar (Jay *et al.*, 2007; Wahlstrom *et al.*, 2003) and birds (Swirski *et al.*, 2014; Wallace *et al.*, 1997). *Salmonella* is rarely isolated from farm animals in Sweden (SVA, 2015), but this bacteria is endemic in wild birds (Taylor & Philbey, 2010; Wahlstrom *et al.*, 2003; Tauni & Osterlund, 2000) and has been isolated from wild boar (Sanno *et al.*, 2014). Pathogenic *Y. enterocolitica* has also been isolated from wild boar (Sanno *et al.*, 2014), while *Campylobacter* spp. have been isolated from several wild animals, including birds, hares and wild boar (Wahlstrom *et al.*, 2003). *Listeria monocytogenes* is ubiquitous in nature, including soil, vegetation and other environmental sources (Haase *et al.*, 2014).

When different environmental parameters in primary production of leafy vegetables and strawberries were studied in five different countries (Belgium, Brazil, Egypt, Norway and Spain), *Campylobacter* spp., *Salmonella* and STEC were found to be present in 19, 3.1 and 1.6 % of irrigation water samples, respectively (Ceuppens *et al.*, 2015), while *Salmonella* and STEC were present in 1.8 and 0.9% of soil samples, respectively.

Sources of water used for irrigation

Irrigation water has been suggested as the major source of enteric pathogen contamination in leafy vegetables (Pachepsky *et al.*, 2011). Surface water originating from lakes, ponds, rivers or streams is usually used for irrigation. Surface water sources have variable levels of contamination, and thus the number and type of microorganisms can vary greatly between sampling occasions. Since cattle may shed STEC, they should not be allowed in close proximity to irrigation sources, which can be achieved by fencing and providing drinking facilities away from the water source. Manure should be stored in a controlled way and should not be applied during irrigation season if this involves run-off to sources of irrigation water (Johannessen *et al.*, 2015). Non-functional wastewater treatment at residential buildings close to water sources also increases faecal contamination, with human pathogens potentially present (Alsanius, 2014).

Distribution of water from source to field

The microbial quality of irrigation water is not only affected by the source, but also by the pipes and equipment used for transporting the water from the source to the field and distributing it to the plants. The biofilms present in pipe-based irrigation systems may affect water quality while water is transported in the irrigation system (Pachepsky *et al.*, 2012). In addition, since water remains in pipes between irrigation events, this may also affect water quality due to growth of bacteria. Therefore, it is important to monitor water quality at the endpoint of irrigation systems in the field, rather than at the intake (Pachepsky *et al.*, 2012). There are systems available for decontamination of irrigation water (Alsanius *et al.*, 2011). To be an efficient tool, such systems should be installed at the end-point of the irrigation system, for example on the irrigation ramp at the field site. In commercial-scale production, irrigation with sprinklers or portable water reel irrigation systems in which water is applied in the form of a spray and reaches the soil more or less like rain are commonly used (Uyttendaele *et al.*, 2015). This facilitates the contamination of leafy vegetables by exposing the edible portions directly to water with potential presence of pathogens (Uyttendaele *et al.*, 2015).

Alternatively, splash by water with good microbiological quality on contaminated soil may also reach edible portions of leafy vegetables.

Guidelines and recommendations on the microbiological quality of irrigation water

Guidelines and standards on the microbiological quality of irrigation water have been established in some countries, but few of these are mandatory (Alam *et al.*, 2014). This was reflected in a study in the U.S. in which only a minority (27%) of growers using surface water for irrigation of fresh fruit and vegetables reported that they tested the water (Bihn *et al.*, 2013). There are currently no mandatory directives regulating the quality of irrigation water in Sweden, but there have been ongoing discussions on stricter guidelines. Almost 10 years ago, it was suggested by the Swedish authorities that irrigation water should at least comply with bathing water quality (max. 100 colony-forming units (CFU) of *E. coli*/mL) and that irrigation ≤48 h prior to harvest should use water of drinking water quality, *i.e. E. coli* should not be detected in 100 mL (Livsmedelsverket *et al.*, 2007). However, in the present Swedish guidelines for primary producers of vegetables (LRF Trädgård, 2014), there are no specified requirements about sampling and microbiological standards of irrigation water. However, it is advised to perform hazard analysis to identify and manage risk factors affecting the quality of irrigation water (LRF Trädgård, 2014). A holding time of 48 hours between irrigation and harvest is advised in the Swedish guidelines for vegetable growers (LRF Trädgård, 2014), as this is believed to reduce the numbers of pathogenic bacteria on the leaves by UV radiation, drying or competition with commensal microbiota (Brandl & Mandrell, 2002). However, concerns have been raised about the recommended length of holding time, since a greenhouse study showed that although there was a reduction in *E. coli* O157:H7, considerable numbers were still present even after a holding time exceeding the recommended 48 h (Alam *et al.*, 2014).

Manure as the source of contamination

Manure and contaminated soil have been identified as sources of leafy vegetable contamination. Manure may be applied as fertiliser to fields used for production of vegetables in both conventional and organic systems, with increased use in organic systems (Johannessen *et al.*, 2004). Proper composting of manure may result in a hygienised product that is safe to use as a soil improver (Nicholson *et al.*, 2005). However, spreading fresh manure in the field poses a risk of pathogen contamination and there should be a sufficient interval between manure application and harvest, particularly for those products that will be consumed

raw, in order to allow any pathogen present to die off. Pathogenic bacteria may survive in soil and manure for long periods. *Escherichia coli* O157, *Salmonella* spp. and *Campylobacter* spp. can survive for up to three months in slurry (manure with high water content) stored at temperatures <20 °C and up to one month after spreading of manure on soil, while *L. monocytogenes* can show even longer survival times (Nicholson *et al.*, 2005). According to the guidelines for primary producers of vegetables in Sweden, untreated manure should not be used during the growing season and if organic fertilisers of animal origin are used, these should be sanitised (LRF trädgård, 2014).

The ability of plants to internalise foodborne pathogens through intact roots during growth has been a debated topic in recent years. As reviewed by Hirneisen *et al.* (2012), in most studies where plants have been grown in inoculated soil, no bacterial internalisation has been detected in edible parts of the plant. For those studies where internalisation was observed in soil-grown crops, it has been sporadic and at low-level (Hirneisen *et al.*, 2012). Erickson *et al.* (2014) concluded that internalisation of *E. coli* O157:H7 seems to be unlikely to occur in germinating seeds in natural field conditions. However, there are other possible routes of pathogen entry into plant tissues, for example via natural pores (*e.g.* stomata) or wounds in the surface of leafy vegetable.

1.6.2 Post-harvest contamination

Processing of leafy vegetables into RTE products, *i.e.* washing and sometimes shredding, causes damage to the external barrier of the leaf. This results in an increased surface area for bacterial contamination and release of exudates that facilitate microbial growth (Brackett, 1994).

Washing

Reasons for washing leafy vegetables are to remove soil, debris or bird droppings and to decrease microbial contamination (Nousiainen *et al.*, 2016). In washing facilities, several washing steps are used before the leafy vegetables are presented to the consumer as RTE.

The production of RTE leafy vegetables world-wide is generally highly mechanised. In brief, leafy vegetables are usually harvested into bins in the field and the bins are placed in refrigerated trucks and transported to a refrigerated processing facility (FAO/WHO, 2008). The washing lines in processing facilities may differ, but they often include a conveyor carrying the leafy vegetables through a shaker to remove foreign material. The leafy vegetables are then washed in different baths and sometime sanitized in a water flume. Excess water is removed, for example with a centrifuge, before packaging.

Use of potable water compared with sanitiser

Use of potable water for washing leafy vegetables reduces the number of bacteria by 0.1-1 $\log_{10}$ units, *i.e.* by 90% at best (WHO & FAO, 1998). However, leafy vegetables with a bacterial population of 10^7 CFU/g will still have at least 10^6 CFU/g after washing with potable water. In many countries, sanitising agents are used during washing of leafy vegetables. Chlorine is the most widely used sanitiser in the fresh produce industry (Oliveira *et al.*, 2015a), and washing with the chlorine concentrations normally used (50-200 ppm) results in a reduction in microorganism levels of 1-2 $\log_{10}$ units, *i.e.* by 99% at best (WHO & FAO, 1998). Leafy vegetables with a bacterial population of 10^7 CFU/g, will thus still have at least 10^5 CFU/g after washing with chlorine. A more efficient washing process for leafy vegetables is difficult to achieve (WHO & FAO, 1998), since indigenous microorganisms attach strongly to the leaf surface (Brackett, 1994) or are protected in biofilms (Gil *et al.*, 2009; Carmichael *et al.*, 1999). Some microorganisms may also adhere to cut surfaces or in stomata and will thus be inaccessible for the wash water (Seo & Frank, 1999).

Even if washing with chlorine is more effective than washing with potable water in reducing the microbial population on leafy vegetables, the main effect of chlorine is to maintain the quality of the wash water and to prevent cross-contamination (FDA, 2008). The sanitiser kills microbes before they attach or become internalised in the leafy vegetable (Gil *et al.*, 2009). It has been concluded by FAO/WHO and the European Food Safety Authority (EFSA) that since use of chlorine reduces cross-contamination of leafy vegetables via wash water, it reduces the risk of exposure of consumers to pathogens (EFSA, 2015; FAO/WHO, 2008). Another advantage is that less water is required in the washing process, since water can be re-used into a higher extent than when using potable water. However, high organic loads, from dirt or debris, can rapidly reduce the effectiveness of sanitisers in preventing cross-contamination and reducing microbial load (FAO/WHO, 2008).

Until recently, washing leafy vegetables with chlorine has not been permitted in Sweden (LRF Trädgård, 2014). However, washing of plant-derived food with chlorine as a 'processing aid', as defined in European Commission (EC) regulation No. 1333/2008 (EC, 2008), can be permitted under national regulations in the European Union (EU), provided that residues in the final product do not present any health risk. The Swedish National Food Agency has recently accepted chlorine as a processing aid when used during washing of leafy vegetables (Livsmedelsverket, 2015), if potable water is used during the last washing step to remove disinfectants (EFSA, 2014). If chlorine residues are still present in the final product, the producer must ensure that the residues are not a health concern for consumers (EC, 2004). It is also emphasised that processing

aids must never compensate for poor hygiene practices (Livsmedelsverket, 2015). To the best of my knowledge, Swedish washing facilities have not yet introduced chlorine in their washing processes of leafy vegetables.

In one Swedish washing facility, the system is filled with potable water at the beginning of each of two shifts every day (Grudén *et al.*, 2016). The leafy vegetables are transported on conveyors through three washes; pre-wash, main wash and final wash. The leafy vegetables are rinsed by sprinkling water while being transported between the pre-wash and main wash tanks and after the final wash. For the main wash, final wash and both rinsing steps, fresh water is introduced into the washing line. Used process water from these steps is reused in the pre-wash after removing sludge, to minimise the volume of wastewater produced. The whole washing line is cleaned once per day, at the end of the second shift (Grudén *et al.*, 2016).

Control of post-harvest processing

All food business operators in the EU have to comply with requirements for good hygiene practice in accordance with Regulation (EC) No. 852/2004 (EC, 2004) on the hygiene of foodstuffs. Strict maintenance of good hygiene practices at all stages of the food chain will prevent contamination of food by pathogens. Establishments other than primary producers and associated activities must implement procedures based on the Hazard Analysis and Critical Control Points (HACCP) principles to effectively control the risks. Distributors and retailers must also guarantee that the cold chains is always maintained, to prevent bacterial growth (EC, 2004, article 1). Regulation (EC) No. 178/2002 (EC, 2002) requires that a food business operator at any point in the food production chain can identify its suppliers and customers, thus enabling traceability and tracking.

In addition to the general hygiene requirements, several microbiological criteria have been laid down in Regulation (EC) No. 2073/2005 (EC, 2005b). Some are food safety criteria for the final product, *e.g. Salmonella* spp. must be absent in RTE vegetables and *L. monocytogenes* level must be less than 100 CFU/g in RTE products during the whole shelf-life. If these criteria are not met, the products are deemed not acceptable for consumption.

Escherichia coli is an indicator of faecal contamination and is included as a process hygiene criterion in EU regulation No. 2073/2005 (EC, 2005b), with a certain level that should not be exceeded during the manufacturing process of RTE vegetables. A process hygiene criterion is guiding and should necessitate improvements in production hygiene and selection of raw materials if unsatisfactory.

1.7 Storage conditions

1.7.1 Temperature and shelf-life

Temperature control and maintenance of adequate cold chain conditions are critical to food safety, as temperature is the single most important factor contributing to bacterial growth and survival (WHO & FAO, 2008). Storage temperatures in retail differ between countries. In Sweden, the law does not regulate storage temperature. Instead, it is up to food producers to decide which storage temperature to recommend for a particular food product (TemaNord, 2016). According to EU Regulation No. 1169/2011 on the provision of food information to consumers (EC, 2011), the storage temperature must be stated on the pack label if a product requires cool storage. Food business operators must ensure that their food products are safe and that they meet food safety criteria throughout their shelf-life under reasonably foreseeable conditions of distribution, storage and use (EC, 2005b).

The storage temperature of refrigerated foods in Nordic countries varies from 3 to 8 °C, with the highest temperature applying in Sweden (TemaNord, 2016). While there is no legal regulation of storage temperature, there are guidance documents published on the website of the Swedish National Food Agency that specify maximum 8 °C as the storage temperature for many refrigerated products (Svensk Dagligvaruhandel, 2013). However, the same website (Livsmedelsverket, 2016a) advises consumers to store refrigerated food products at 4 to 5 °C to extend durability and reduce food waste. For RTE salad with chicken, the maximum recommended temperature is 4 °C in Norway, 5 °C in Denmark and 6 °C in Finland (TemaNord, 2016). In Sweden, maximum 8 °C is usually indicated on the pack label of RTE salads with chicken (Paper I). These products often have a shelf-life of three days and a 'best-before' label stating the period in which the producer guarantees that a certain food item can be expected to retain its original condition in unopened packaging (TemaNord, 2016). The U.S. Food and Drug Administration (FDA) recommends a storage temperature below 5 °C for RTE leafy vegetables (FDA, 2010). The recommended storage temperature for RTE leafy vegetables in Sweden is often displayed on the product label as maximum 4 °C, but it may also be displayed as maximum 5 or 8 °C, depending on the brand (Testfakta, 2014). Bags of RTE leafy vegetables in Sweden are generally labelled with a 'best-before date', with a shelf-life of approximately 7-9 days.

To reduce food waste, in 2015 a Swedish food industry working group started work with the aim of lowering the storage temperature in the cold chain in Sweden by 2020 (TemaNord, 2016). A reduction in storage temperature would

also have benefits in terms of quality and safety. For example, a reduction from 8 to 4 °C extends the time to expiry date by about a week. Furthermore, *L. monocytogenes* multiplies much more slowly at 4 °C than at 8 °C (TemaNord, 2016).

Regardless of the recommended storage temperature, many studies report temperature abuse in both retail (Koutsoumanis *et al.*, 2010) and domestic refrigerators (EFSA, 2012). Following a Swedish survey in 2011 that involved almost 2,000 Swedish school-children (Marklinder & Erikkson, 2015), it was concluded that more than half (58%) of home refrigerators had an air temperature that was higher than 8 °C in at least one spot (Mattias Eriksson, Swedish University of Agricultural Sciences, personal communication 2015). In an U.S. study, 20% of domestic and commercial refrigerators were found to operate at a temperature higher that 10 °C (Jol *et al.*, 2005).

1.7.2 Leafy vegetables compared with ready-to-eat leafy vegetables

Under Swedish regulations, only leafy vegetables that have been cut or shredded or are presented to the consumer as RTE have to be labelled with a recommended storage temperature and a best-before date (Livsmedelsverket, 2004). For leafy vegetables (mainly baby leaves) that have not been washed or cut, but which are packed in bags typical of RTE products, storage instructions may be absent or vague, for example indicated as ‘should be stored cool’. These products are sometimes displayed at room temperature, as observed in supermarkets in Sweden (Figure 4). In a study in Finland, many packs of leafy vegetables at retail showed unsatisfactory information on recommended storage temperature, with almost 40% of packages lacking any information on recommended storage temperature (Nousiainen *et al.*, 2016). This can lead to temperature abuse in storage, thus influencing the bacteriological quality and safety of these products. Moreover, almost 30% of packages of bagged leafy vegetables in that study did not show information on whether the product was washed or not (Nousiainen *et al.*, 2016). Since leafy vegetables often appear ‘clean’ even if they have not been washed, some consumers may assume that bagged leafy vegetables in general are RTE. This may result in consumption of the product without any risk-reducing washing step (Ottoson *et al.*, 2011), either in washing facilities during production or in the consumer’s home.

Figure 4. Bags of mixed leafy vegetables that are not ready-to-eat, *i.e.* the leaves have not been washed. The bags are displayed at room temperature in a Swedish supermarket (photo by author).

1.7.3 Metabolic activity and packaging of leafy vegetables

Packaging is applied to ease product handling in a hygienic way and also prevents water loss from the leafy vegetables. The storage temperature should control both enzymatic activity and growth of naturally present microbiota in leafy vegetables. While photosynthesis stops when leafy vegetables are harvested and the connection to the root system is broken, there is still some metabolic activity in the leaves, although it depends on stored energy and water. These processes are gradually reduced until the vegetables are decayed (Andersen & Risum, 1993). After harvest, leafy vegetables respire, *i.e.* consume O_2 and produce CO_2. The rate of respiration is increased by mechanical or microbiological damage to the leaves and is usually highest immediately after harvest, which causes damage to the leaves. Respiration also increases with increased storage temperature (Andersen & Risum, 1993). Because of this respiration, the gas atmosphere is always modified if leafy vegetables are sealed in a package, resulting in increased CO_2 levels and reduced O_2 levels (Andersen & Risum, 1993).

Polymeric films with a wide range of gas diffusion characteristics are used to passively alter the gas composition surrounding RTE leafy vegetables in order to obtain an optimal gas composition for specific varieties (Gil *et al.*, 2015). These gas levels, combined with refrigeration, slow down product respiration and microbial growth, delay physiological ageing and thus extend shelf-life. However, concerns have been raised about leafy vegetables packaged under

these modified gas conditions, since prolonging the shelf-life will increase the time available for pathogen growth, especially under temperature abuse (Francis *et al.*, 1999). Many pathogens are facultative anaerobes and may not be greatly affected by the modified atmosphere, while some natural competitors of pathogens may be inhibited (Francis *et al.*, 1999). There are conflicting data on whether modified atmosphere packaging (MAP) affects growth of foodborne pathogens in leafy vegetables, with studies showing no effect currently dominating (as reviewed by Oliveira *et al.*, 2015b). For example, no difference in growth of *L. monocytogenes* or *E. coli* O157:H7 on romaine lettuce has been found during storage in modified atmosphere compared with air conditions at 5 or 25 °C (Oliveira *et al.*, 2010).

1.8 Consumer and food handler behaviour and ready-to-eat leafy vegetables

1.8.1 Is an additional wash of ready-to-eat leafy vegetables necessary?

Consumers' main reason for rinsing leafy vegetables (that have not already been washed and packaged) may be to ensure that pesticide residues are removed (Leifert *et al.*, 2008). Consequently, these consumers may be less likely to rinse organically grown vegetables, although these may be contaminated with human pathogens from manure and/or irrigation water.

Consumers in the U.S. are advised to rinse leafy vegetables in order to reduce the risk of foodborne disease, but only when leafy vegetables have not already been packaged and labelled as RTE (Fight Bac!, 2016b). A panel of Northern American scientists with expertise in microbial safety of RTE fruit and vegetables has concluded that RTE leafy vegetables produced in a facility inspected by a regulatory authority and operating under good manufacturing practice (GMP) do not need additional washing at the time of use (Palumbo *et al.*, 2007). If harmful microorganisms are present after commercial washing treatments, they are likely to resist removal or inactivation by further washing. In addition, improper handling in the home or restaurant during additional washing may increase the risk of cross-contamination from food handlers and food contact surfaces (Palumbo *et al.*, 2007). If the end-user chooses to wash RTE leafy vegetables before use, they should protect the product from cross-contamination from raw foods, contaminated equipment or inadequately washed hands (Fight Bac!, 2016a). They should wash their hands thoroughly for 20 s with soap and warm water before handling RTE leafy vegetables and rewash when necessary. The sink and any utensil that comes into contact with the

product should be sanitised and cold, running tap water should be used to wash RTE leafy vegetables (Fight Bac!, 2016b). It is doubtful whether these hygiene instructions will actually be followed by most consumers, and in fact a consumer survey in the U.S. showed that almost 50% of respondents did not wash their hands before handling fresh fruit and vegetables (Li-Cohen & Bruhn, 2002).

2 Aims of the thesis

The overall aim of this thesis was to answer the question 'Is your salad safe to eat?' Specific objectives were to:

- Determine the microbial safety of pre-packed RTE mixed-ingredient salads containing cooked chicken, ham or smoked salmon, by investigating the occurrence of the foodborne pathogens *L. monocytogenes*, STEC, pathogenic *Y. enterocolitica*, *Salmonella* spp. and *Campylobacter* spp.

- Examine the fate of *L. monocytogenes*, pathogenic *Y. enterocolitica* and *E. coli* O157:H7 *gfp*+ inoculated in low numbers into RTE baby spinach and mixed-ingredient salads stored at refrigerator temperature (8 °C) or temperature abuse conditions (15 °C) during shelf-life and to explore the food safety implications of the results

- Compare the microbiota of RTE baby spinach and mixed-ingredient salads before and after seven days of storage at 8 °C or 15 °C and investigate whether bacterial pathogens can be detected using the 16S rRNA amplicon dataset

- Explore associations between bacterial communities and the foodborne pathogens *L. monocytogenes,* pathogenic *Y. enterocolitica* and pathogen model organism *E. coli* O157:H7 *gfp*+ when experimentally inoculated into salads before storage

3 Considerations on materials and methods

This section provides a summary of the materials and methods used in Papers I-III, with the focus on methodological considerations. A key consideration and challenge was to enable inferences relevant to consumer protection and food safety. Detailed descriptions of the procedures performed are presented in the individual papers.

3.1 Materials chosen for the studies

The RTE mixed-ingredient salads analysed in Paper I were collected mainly from supermarkets, but also from convenience stores in the city of Uppsala. The sampling was based on observations of the supply of these salads on retail level, to represent the most common types of mixed-ingredient salads available to consumers. Of these, three categories of salads with different protein sources were chosen for the study. All were packed in plastic bowls with lids and, with few exceptions, had been prepared by a manufacturer. The recommended storage conditions indicated on the label of most salads were maximum 8 °C for three days. To reflect the microbiological quality of salads that had been stored accordingly, the aim was to start analyses on the last day of shelf-life. Thus, any *L. monocytogenes* and pathogenic *Y. enterocolitica* present, which are able to grow at refrigerator temperature, would have had a chance to grow.

Figure 5. Sample preparation from one salad package by picking different ingredients to obtain a representative sample of the salad components (photo by author).

In Paper II, growth of inoculants was studied in two different sample types of RTE salad. One sample type consisted of plain leafy vegetables and the other represented a mixed-ingredient salad, with addition of a meat ingredient to the leafy vegetable ingredient. Since chicken was found to be the most common protein ingredient in the mixed-ingredient salads analysed in Paper I, it was the meat ingredient chosen for Paper II. Ready-to-eat baby spinach was chosen to represent plain leafy vegetables, based on the fact that it is one of the most popular RTE bagged salad products at retail in Sweden. Ready-to-eat baby spinach was also chosen as the leafy vegetable ingredient in the mixed-ingredient salad samples analysed in Paper II. This was despite the fact that, based on findings in Paper I, iceberg lettuce is the most common leafy vegetable ingredient in mixed-ingredient salads and is also present in larger proportions than baby spinach in these salads. However, iceberg salad is mainly present in mixes of different leafy vegetables in RTE bagged salad products at retail in Sweden and is thus not representative of the plain leafy vegetable sample type. The choice to include baby spinach rather than iceberg lettuce was not expected to affect pathogen growth, since *L. monocytogenes* has been reported to grow equally on iceberg lettuce and baby spinach during storage at 7 °C (Lokerse *et al.*, 2016).

3.2 Methods used for isolation or detection of pathogens

In Paper I, in which the microbiological quality of RTE mixed-ingredient salads was studied, standard isolation methods (NMKL or ISO) were used for the detection of *Campylobacter* spp., *Salmonella* spp. and *L. monocytogenes.* Polymerase chain reaction (PCR)-based methods were used for the detection of pathogenic *Y. enterocolitica* and STEC. Some of the methods are commented below.

3.2.1 Shiga toxin-producing *Escherichia coli*

It is challenging to isolate STEC from food samples (Rivas *et al.*, 2015). Therefore, a PCR method for the detection of shiga-like toxin (*stx*) 1 and *stx*2, the main virulence factors of STEC, was used in Paper I for an initial screening of samples. Samples that tested positive for stx_1 and/or stx_2 were further screened by PCR for *eae* (intimin), which is the gene associated with the attaching and effacing lesion of enterocytes (EURL VTEC, 2013), and for the serotypes O157, O26, O111, O103, O145, which are serotypes causing the majority of haemolytic uremic syndrome (HUS) cases in Europe (EURL VTEC, 2013). Procedures for isolation of strains were available for samples that tested positive for *eae* and any of these five serotypes. A challenge is that very few isolates are generally obtained with available methods although samples have tested positive in PCR screening (Rivas *et al.*, 2015). Without an isolate, it is not possible to determine whether the various genes detected in a sample derive from single potentially pathogenic cells or from different non-pathogenic cells. Another limitation of this method is that it only focuses on *eae*-positive strains with any of five specific serotypes. Approximately 10% of the strains that caused enterohaemorrhagic *E. coli* (EHEC) cases in the EU in 2007-2010 did not carry the *eae* gene (EFSA, 2013a) and samples collected during the large STEC outbreak in 2011, with sprouts as the most likely vehicle (Buchholz *et al.*, 2011), did not test positive for *eae* or for any of the five serotypes included in standard methods (EFSA & ECDC, 2013).

3.2.2 Listeria monocytogenes

In Paper I, ISO 11290-1:1996 (ISO, 1996) was used for qualitative analysis of *L. monocytogenes.* In this method, 25 g of food sample were subjected to enrichment and, in theory, only one *L. monocytogenes* bacterium in this 25 g of sample is enough for a positive result. The isolated strains were subjected to pulsed-field gel electrophoresis (PFGE) after being mixed with the restriction enzymes AscI and ApaI to enable comparison of the profiles with those from

human cases registered by the Public Health Agency of Sweden and to find potential temporal relationships.

3.2.3 Pathogenic *Yersinia enterocolitica*

A TaqMan PCR-based method (Lambertz *et al.*, 2008) was used in Paper I for the detection of *ail*, which is a gene for the attachment invasion locus located in the chromosome of pathogenic *Y. enterocolitica.* Although the *ail* gene is largely restricted to pathogenic serotypes of *Y. enterocolitica*, a variant may be found in a small proportion of biotype 1A strains (Sihvonen *et al.*, 2011). Since biotype 1A strains are generally considered non-pathogenic (Grant *et al.*, 1998), these strains may be associated with false positive results.

When pathogenic isolates occur in food samples, they are usually present only at low concentrations in a complex background microbiota (Petsios *et al.*, 2016). Even when present in higher concentrations, the microbiota of some food products, *e.g.* leafy vegetables, may overgrow pathogenic *Y. enterocolitica* on the agar medium used (see below). Thus, pathogenic *Y. enterocolitica* strains are difficult to isolate from food even if they are present (Fredriksson-Ahomaa & Korkeala, 2003). For that reason, several methods were used in Paper I, including additional protocols in the updated ISO method (ISO, 2015), in an attempt to maximise recovery of isolates from *ail*-positive samples, as outlined in Figure 6.

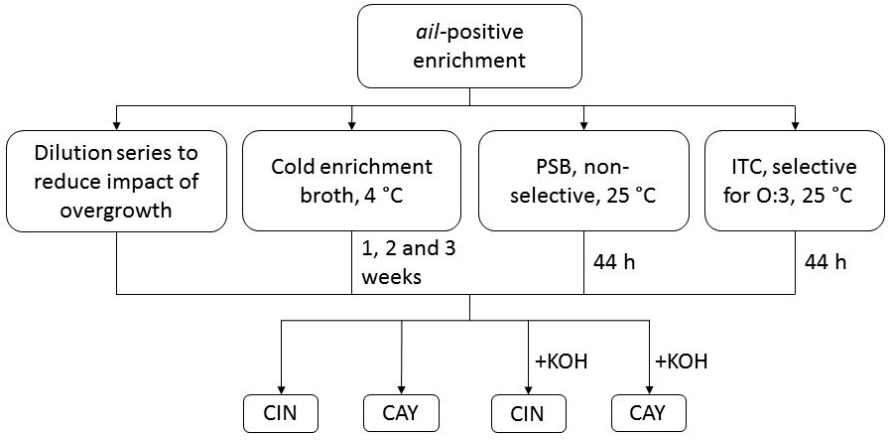


Figure 6. Overview of the culture methods used in Paper I for samples that were *ail*-positive but culture-negative in the TaqMan PCR-based method.

The use of cefsulodin-irgasan novobiocin (CIN) agar is mandatory in the ISO method (ISO, 2015), while the use of CHROMagar Yersinia (CAY) is optional. Both were used in Paper I, to increase the chances of isolation. Several other bacteria apart from pathogenic *Y. enterocolitica* can grow on CIN agar medium with a similar appearance as the target pathogen, which makes the selection of presumptive pathogenic *Y. enterocolitica* difficult. CAY has been available for some years and is a selective chromogenic agar for presumptive pathogenic *Y. enterocolitica* colonies. However, CAY was developed for the detection of pathogenic *Y. enterocolitica* in stool samples and has not yet been routinely used and evaluated for the detection of the pathogen in food (Petsios *et al.*, 2016). Potassium hydroxide (KOH) treatment prior to streaking on selective agar media can reduce the levels of competing microorganisms, since pathogenic *Y. enterocolitica* is more tolerant to alkaline conditions than most other bacteria (Aulisio *et al.*, 1980). However, since the number of pathogenic *Y. enterocolitica* may also be reduced during this treatment and since KOH treatment dilutes the enrichment (ISO, 2015), non-treated enrichment was also streaked.

3.3 Inoculated strains in Papers II and III

Since salads have high levels of background microbiota, it was necessary to use labelled inoculated strains to enable identification in Papers II and III. In Paper II, growth of *L. monocytogenes*, pathogenic *Y. enterocolitica* and *E. coli* O157:H7 was tested and samples from these growth trials were further analysed in Paper III. To account for variations in growth among different strains, use of a cocktail of several strains for growth trials is often recommended (EURL Lm, 2014; Beuchat *et al.*, 2001). In Papers II and III, the performance of single strains of each pathogen or pathogen model organism was studied, but with recognition that other strains could have been more or less tolerant to the test conditions. It would have been preferable to use strains that had originally been isolated from leafy vegetables or mixed-ingredient salads or from patients suffering from foodborne disease caused by contamination of these food products. However, such strains were not available at the time of the inoculation trials.

3.3.1 *Escherichia coli* O157:H7 *gfp*+

For practical and safety reasons, an *eae*-positive but stx_1/stx_2-negative strain of *E. coli* O157:H7 that was already available at Swedish University of Agricultural Sciences (SLU) was used. This strain is resistant to ampicillin (100 µg/mL) and is labelled with green fluorescent protein (*gfp*) which can be induced to fluoresce in UV-light (Alam *et al.*, 2014). To reduce background microbiota, agar plates

in Paper II were supplemented with ampicillin (Figure 7). The *E. coli* O157:H7 *gfp*+ strain has shown similar growth rates to the wild type *in vitro* and on plants, and has been used in previous studies performed at SLU (Boqvist *et al.*, 2015; Alam *et al.*, 2014; El-Mogy & Alsanius, 2012). Gfp labelling of *E. coli* O157:H7 has been reported to be a stable surrogate for safety-related studies and the plasmid has been shown to have an insignificant effect on bacterial growth (Ma *et al.*, 2011). Others have reported that *gfp*-labelled bacterial cells remain fluorescent following stress and remain detectable in all growth phases (Lowder *et al.*, 2000; Tombolini *et al.*, 1997).

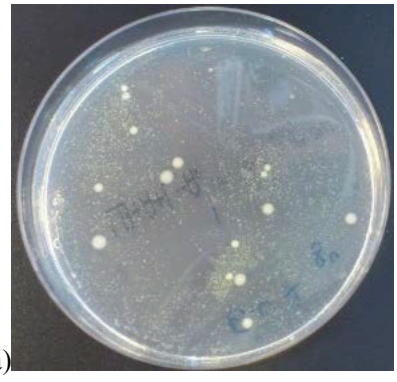
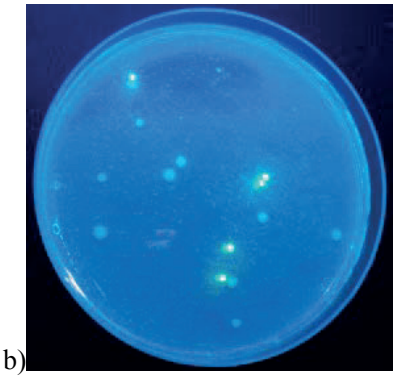
a)  b) 

Figure 7. (a) The ampicillin concentration present in agar medium inhibited some, but not all, background microorganisms, and thus ampicillin resistance alone would not have been enough for identification. (b) In this agar plate, five of the colonies were induced to fluoresce in UV-light and were thus identified as *E. coli* O157:H7 *gfp*+ (photo by author).

3.3.2 Strains of pathogenic *Yersinia enterocolitica* and *Listeria monocytogenes*

The pathogenic *Y. enterocolitica* and *L. monocytogenes* strains were chosen based on the following criteria: the strain had to be available from the Culture Collection University of Gothenburg, Sweden (CCUG); the strain had to be characterised and shown to be stable over time; and the strain had to be originally isolated from food. The selected strains were reference strains at the Swedish National Food Agency. A *Y. enterocolitica* 4/O:3 strain was used, since it is the most common bioserotype causing yersiniosis in Sweden (Socialstyrelsen, 2013). However, in recent outbreaks of yersiniosis associated with leafy vegetables, serotype O:9 has been responsible (MacDonald *et al.*, 2016; MacDonald *et al.*, 2011), which may indicate that O:9 is more commonly associated with RTE salad than O:4.

The *L. monocytogenes* and pathogenic *Y. enterocolitica* strains were cultured on tryptic soy agar (TSA) plates with stepwise increasing concentrations of

rifampin until the strain was finally resistant to 200 µg/mL rifampin, which was the concentration used in agar medium to inhibit background microbiota. Lower concentrations of rifampicin (100 µg/mL) have been reported to completely inhibit growth of background microbiota in samples of romaine lettuce and spinach (Fishburn *et al.*, 2012). However, in Paper I a low frequency of colonies with different morphology than the targets still appeared at rifampicin concentrations of 200 µg/mL. A selection of these background isolates were analysed by MALDI-TOF Mass Spectrometry (performed at the Swedish National Veterinary Institute, SVA) for identification. Six of 16 isolates could not be identified, while five were found to be isolates of the genera *Pantoea, Pseudomonas*, *Erwinia* and *Lactobacillus* and another five isolates were identified as yeast (*Candida*).

The growth ability of the final strains of *L. monocytogenes* and pathogenic *Y. enterocolitica* was tested against that of the wild-type strains. The rifampicin-resistant strains showed growth rates essentially equal to those of the parent strains when optic density (OD620) and CFU levels were compared *in vitro* during growth in medium at 15 °C until stationary phase.

3.3.3 Inoculation levels and storage conditions

In Paper II, the strains were inoculated at a level of 50-100 CFU/g. This level was a compromise between the aim for an inoculation level that mimicked realistic contamination conditions and a level enabling inoculation and enumeration during the trials. It has been estimated that natural contamination of *L. monocytogenes* on fresh produce occurs at levels between 0.1 and 1 CFU/100 g (Crepet *et al.*, 2007), which is much lower than the inoculation levels in Paper II. However, 50-100 CFU/g enabled enumeration and is much lower than the 10 000-100 000 CFU/g used in other inoculation studies (Khalil, 2016; Bovo *et al.*, 2015). In addition, initial levels of 50-100 CFU/g can be related to the EU microbiological criterion for *L. monocytogenes*, whereby concentrations must not exceed 100 CFU/g during shelf-life (EC, 2005b).

Storage temperatures of 8 and 15 °C were chosen for the growth trials, since 8 °C is the recommended maximum temperature indicated on most refrigerated food products in Sweden and 15 °C is a temperature exemplifying temperature abuse conditions.

3.4 Risk assessment in Paper II

In the dose-response models in Paper II, different serving sizes were used for RTE baby spinach (30 g) and mixed-ingredient salad (300 g). This was based on

the assumption that 30 g of RTE baby spinach would occupy 40-50% of the plate (Figure 8), which is the proportion of a healthy meal that is recommended to consist of vegetables according to nutrition recommendations by the Swedish National Food Agency (Livsmedelsverket, 2016c). Mixed-ingredient salad, on the other hand, is intended to be consumed as a full meal. In Paper I, the packs of mixed-ingredient salads sampled ranged in weight from 310 to 550 g. However, while some mixed-ingredient salads mainly consist of leafy vegetables and chicken, *e.g.* Caesar salad, they are more likely to contain additional ingredients, such as other raw vegetables (*e.g.* tomatoes) or carbohydrate sources (*e.g.* pasta). Therefore, a slightly smaller serving size (300 g) was used in the models in Paper II.

Figure 8. One serving of ready-to-eat (RTE) baby spinach (left) based on nutrition recommendations by the Swedish National Food Agency, which are illustrated here for physically active (middle) and non-active (right) individuals. Illustrations from (Livsmedelsverket, 2016c), photo by author.

Risk assessment modelling was performed for *L. monocytogenes* and *E. coli* O157:H7 but not for pathogenic *Y. enterocolitica* since there was no applicable dose-response model available for the latter. Although risk is defined as the probability times the consequences of a hazard, in Paper II the consumer risk measured as probability of infection was used as a simplification. In the stochastic model approach, the risk was presented with the 5th to 95th percentile, after using the Monte Carlo simulation software @Risk (© Palisade Corp.) to capture as much as possible of the variability and uncertainty.

3.5 16S rRNA amplicon sequencing to study microbiota

The samples on day 0 and 7 in the growth trial in Paper II were further analysed by 16S rRNA amplicon sequencing (Illumina MiSeq) in Paper III to explore the microbiota of baby spinach and mixed-ingredient salad during cold storage. To enable identification of the inoculated strains in the microbiota, the 16S rRNA genes of inoculants were also amplified. To explore associations between

microbiota and foodborne pathogen contaminants, results from Paper II, based on cultural methods, were combined with results based on Illumina16S rRNA amplicon sequencing. Since there is no standard approach to evaluating associations between growth of inoculated strains and microbiota composition, different approaches were used. To predict the correlation between abundances of different orders (X) and viable inoculate counts (Y), partial least square (PLS) modelling (Eriksson *et al.*, 2006) was performed. Spearman rank sum correlations were also estimated between viable counts of each inoculated strain and changes in sample microbiota from day 0 and 7. In Paper III, identification to genus level was generally feasible, but not identification to species level because of the incomplete 16S rRNA sequence. Statistical analyses were performed at order level and not at genus level. The main reason for this was that most orders were represented by mainly one genus, for example Pseudomonadales was mainly represented by *Pseudomonas*. However, a few orders contained several genera with different temporal dynamics and most likely also other features, for example Enterobacteriales was represented by *Erwinia* and unclassified *Enterobacteriaceae*. Despite this, analyses at the order level were still able to reveal major changes in the microbiota with a manageable number of variables to analyse.

4 Main results and discussion

4.1 Microbiological quality of pre-packed ready-to-eat mixed-ingredient salads

Paper I showed that almost 10% of RTE mixed-ingredient salads analysed were contaminated with foodborne pathogens or presumptive pathogens. This finding was based on both cultural methods and PCR methods detecting pathogenic markers. However, actual presence of a pathogenic isolate, namely *L. monocytogenes*, was only verified in two of the 141 samples.

4.1.1 Listeria monocytogenes

Listeria monocytogenes was isolated from 1.4% (2/141) of the mixed-ingredient salads tested (Paper I). This is comparable to the prevalence reported in studies in Ireland and the U.K. in 2005, in which 2.7 and 4.8% of salads, respectively, contained *L. monocytogenes* (Little *et al.*, 2007; Anonymous, 2005). One of the contaminated salads in Paper I was a smoked salmon salad, which contained both hot-smoked and cold-smoked salmon and had been prepared on-site in a supermarket. Additional ingredients in this salad were pasta, prawns, crayfish, tomatoes, sweetcorn, cucumber, iceberg lettuce and parsley. The number of salmon salads included was low in both Paper I and the U.K. study, but *L. monocytogenes* was present in 8% (1/12) of the RTE salads containing salmon in Paper I and in 10% of the salmon samples in the U.K. study (Little *et al.*, 2007). Smoked or gravad salmon is considered an important vehicle of foodborne human listeriosis (EFSA & ECDC, 2015) and *L. monocytogenes* was detected in 12% of smoked salmon samples at retail in Sweden in 2010 (Lambertz *et al.*, 2012). Therefore, it is not unlikely that the smoked salmon was

the source of *L. monocytogenes* contamination in the smoked salmon salad analysed in Paper I.

Listeria monocytogenes was also isolated from 1% (1/94) of the RTE salads containing chicken. The chicken ingredient in the contaminated salad in Paper I consisted of cubes of cooked meat. The other ingredients were iceberg lettuce, sweetcorn, pineapple, tomatoes, pepper, orange and parsley. Chicken is heat-treated before consumption and this treatment normally eliminates pathogens. However, *L. monocytogenes* may be present in the food processing environment (Moretro & Langsrud, 2004) and thus cross-contamination can occur during processes following heat treatment, *via* for example equipment and human handling. In the U.K. study of mixed-ingredient salads, *L. monocytogenes* was present more frequently in salads containing chicken (6.2%) or chicken and bacon (8.1%) than in salads containing other meat types (*e.g.* ham and beef) (Little *et al.*, 2007), which suggests that chicken may be associated with higher levels of contamination. However, *L. monocytogenes* was detected in only 1.6% of the RTE chicken meat analysed in the EU in 2013 (EFSA & ECDC, 2015), which is not a higher prevalence than reported for other common salad ingredients. For example, *L. monocytogenes* has been found in 3% of RTE salads with no meat ingredient (Sagoo *et al.*, 2003a) and in 1% of samples of ham (Lambertz *et al.*, 2012; Wong *et al.*, 2005). It appears that many ingredients in a mixed-ingredient salad may be contaminated, and thus the risk of contamination increases with the number of added ingredients in a mixed-ingredient salad. No temporal relationships were found between the ApaI/AscI-profiles of *L. monocytogenes* isolates found in Paper I and those from human cases registered by the Public Health Agency of Sweden.

A qualitative method was used in this thesis for the detection of *L. monocytogenes* and thus the *L. monocytogenes* levels in the positive samples were not determined. The EU microbiological criterion for *L. monocytogenes* is ≤100 CFU/g for RTE products on the market, which is considered to pose a negligible risk to a healthy population (EFSA & ECDC, 2015; EC, 2005b). In general, RTE foods have been reported to rarely exceed this level (EFSA & ECDC, 2015), a claim supported by studies in the U.K. and Ireland in which only 0.1-0.2% of RTE salads exceeded the microbiological criterion (Little *et al.*, 2007; Anonymous, 2005). However, even when present in initial numbers <100 CFU/g, *L. monocytogenes* may increase to high numbers if stored under temperature abuse (see section 4.2).

4.1.2 Pathogenic *Yersinia enterocolitica*

Seven (5.0%) of the 141 salad samples analysed in Paper I, all containing cooked chicken as one of the ingredients, tested positive for the *ail* gene in the PCR assay. One *ail*-positive strain was isolated from the initial enrichments included in the PCR-based method, after streaking KOH-treated enrichment on CIN agar. Despite attempts to maximise the chances of harvesting pathogenic isolates from the other *ail*-positive sample enrichments, no other strain was isolated. The *ail*-positive isolate was a biotype 1A strain, which is generally considered to be non-pathogenic. In studies of lettuce samples in Finland and Norway using PCR assays, 2-3% of samples tested positive for pathogenic *Y. enterocolitica.* However, no pathogenic strains were actually isolated in those studies (Johannessen *et al.*, 2002; Fredriksson-Ahomaa *et al.*, 2001). In a recent study from in Finland, 13% of bagged vegetable salads were found to contain *Y. enterocolitica*, but all these cases were biotype 1A (Nousiainen *et al.*, 2016). One *ail*-positive biotype 1A strain was also isolated in that study. The use of PCR for detecting the *ail* gene has also revealed biotype 1A strains in other studies (Kraushaar *et al.*, 2011; Sihvonen *et al.*, 2011; Cheyne *et al.*, 2010). Even if *Y. enterocolitica* biotype 1A strains are generally considered non-pathogenic, this is debated. There have been studies suggesting that this biotype may be associated with disease in humans (Fredriksson-Ahomaa *et al.*, 2012; Huovinen *et al.*, 2010; Burnens *et al.*, 1996; Morris *et al.*, 1991) and genes encoding known and suspected virulence-associated determinants have been found in biotype 1A (Batzilla *et al.*, 2011), indicating their ability to establish infection in immunosuppressed patients.

4.1.3 Shiga toxin-producing *Escherichia coli*

Three salad samples (2.1%) in Paper I tested positive for stx_1 and/or stx_2 in the PCR assay. Two of these salads contained chicken and one contained ham. Only one sample (a chicken salad) that was positive for stx_1 and stx_2 also tested positive for *eae* and one of the five serotypes (O157) in the subsequent PCR analyses. Since the results indicated low numbers of bacteria, no attempt was made to harvest isolates due to minimal chances of success. Without an isolate, it is difficult to interpret the PCR-positive results. In an Irish study of 247 samples of RTE leafy vegetables and herbs, STEC was not detected (Food Safety Authority of Ireland, 2015). In a Swedish study of 630 leafy vegetable samples, 11 samples tested positive for stx_1 or stx_2 and four of these also tested positive for *eae*, but no isolate could be harvested (Livsmedelsverket, 2014).

4.1.4 *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli* as an indicator of faecal contamination

Salmonella spp., *Campylobacter* spp., and thermotolerant coliform bacteria were not isolated from any sample in Paper I. These results were in agreement with those reported by others, *e.g. Salmonella* spp. was not detected in an Irish study of 714 mixed-ingredient salads (Anonymous, 2005) and neither *Salmonella* spp. nor *Campylobacter* spp. was detected in an U.K. study of salads without a meat ingredient (Sagoo *et al.*, 2003a). However, *E. coli* was present in 7% of samples in the latter study, while not detected in Paper I, which may indicate better hygienic conditions during preparation of the RTE salads evaluated in Paper I. In a Swedish study of leafy vegetables, *E. coli* was detected in 37% of samples (Livsmedelsverket, 2014), but the samples in that study were mainly leafy vegetables that had not been washed before analysis.

4.2 Growth of pathogens and pathogen model organism in ready-to-eat salads and implications for food safety

Paper II showed that mixing RTE baby spinach with cooked chicken strongly influenced the populations of inoculated strains during storage, with significant growth of all inoculants in mixed-ingredient salad at 15 °C and of *L. monocytogenes* at 8 °C (Figure 9). In plain baby spinach, growth was observed for *L. monocytogenes* and pathogenic *Y. enterocolitica* only during storage at 15 °C, but their growth was limited compared with that in samples where baby spinach was mixed with chicken. Similar results have been reported by Bovo *et al.* (2015), who demonstrated no growth of *Salmonella enterica* in romaine lettuce at 6 or 14 °C but considerable growth after addition of chicken and storage at 14 °C.

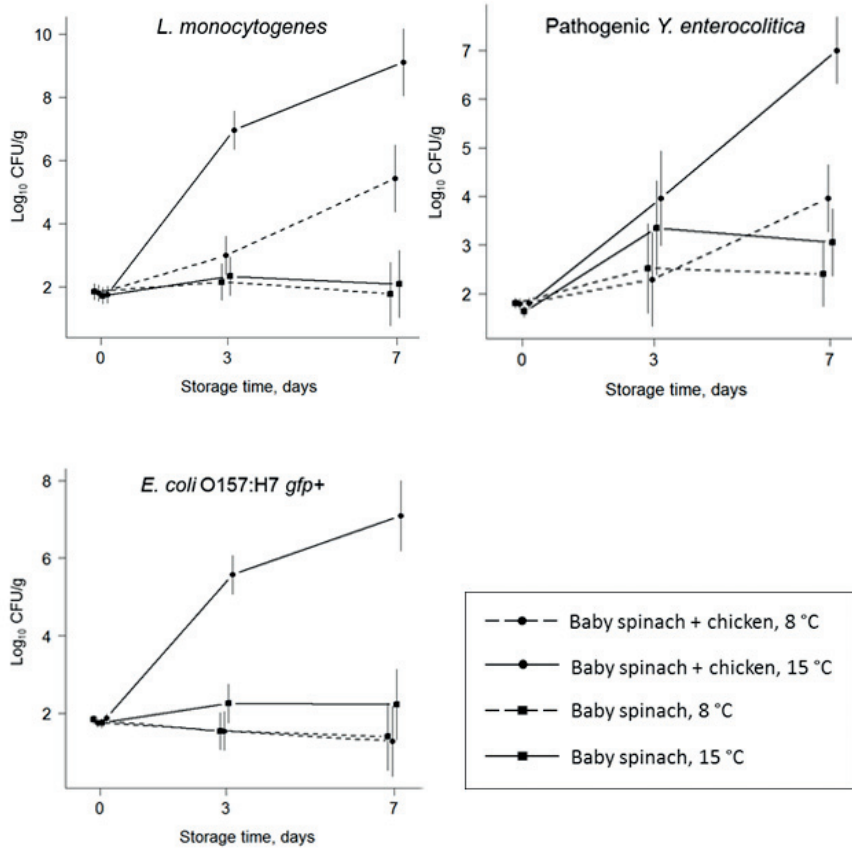


Figure 9. Populations (log_{10} colony-forming units (CFU)/g) of *Listeria monocytogenes*, pathogenic *Yersinia enterocolitica* and *Escherichia coli* O157:H7 *gfp+* in ready-to-eat (RTE) baby spinach or baby spinach mixed with cooked chicken.

4.2.1 Relating growth potential of *Listeria monocytogenes* to EU microbiological criteria for ready-to-eat food

Based on the results in Paper I and in studies from the U.K. and Ireland (Little *et al.*, 2007; Anonymous, 2005), *L. monocytogenes* can be expected to be present in a few per cent of mixed-ingredient salads at retail. Since *L. monocytogenes* is able to grow during cold storage, it is important to study growth at relevant storage conditions. In Sweden, 8 °C is the recommended maximum storage temperature for this product and Paper II showed that significant growth can occur at this temperature during shelf-life, with numbers increasing by approximately 1 log CFU/g during three days. *Listeria monocytogenes* was the

inoculant that reached the highest concentrations during storage, with approximately 7.0 log CFU/g in mixed-ingredient salads after a normal shelf-life of three days under temperature abuse (15 °C). Food products can be considered to support microbial growth when bacterial numbers increase by more than 0.5 log CFU/g during shelf-life (EURL Lm, 2014). However, when interpreting the microbiological criteria in EC Regulation No. 2073/2005 (EC, 2005b), RTE products with a shelf-life of less than five days, for example mixed-ingredient salads, are classified in the category of RTE foods *unable* to support growth of *L. monocytogenes*. For RTE foods able to support growth, *L. monocytogenes* must be absent before the product leaves the food business operator (EC, 2005b), while for RTE food unable to support growth no testing is needed to show absence at this point. For both categories, however, the level of *L. monocytogenes* should be less than 100 CFU/g during its shelf-life. Manufacturers preparing mixed-ingredient salads should be made aware that the length of shelf-life and the maximum storage temperature (8 °C) they recommend for this product actually supports growth of *L. monocytogenes*. Therefore they should control the ingredients and final product before entering the market to minimise contamination by this pathogen. In addition, the results in Paper II suggest that 8 °C is not an appropriate storage temperature for this product, since it does not inhibit growth of *L. monocytogenes*.

4.2.2 Risk modelling of *Listeria monocytogenes*

To explore the food safety implications of these findings, bacterial numbers were translated into risk of infection by modelling. Since the risk of acquiring invasive listeriosis varies greatly between different individuals, modelling was performed for three different population subgroups with differing susceptibility to infection according to Pouillot *et al.* (2015). These subgroups were: healthy adults (<65 years old), the elderly (≥65 years of age) and individuals with solid organ transplants and thus strongly immunocompromised.

For plain baby spinach that had been inoculated with *L. monocytogenes* and stored for 7 days, the estimated median risks of listeriosis were <1 per million servings for all subgroups, regardless of whether the baby spinach was stored at 8 or 15 °C during its shelf-life. However, for inoculated mixed-ingredient salad that had been stored for three days, which is the recommended shelf-life of this product, the risks of listeriosis varied between subgroups and storage temperatures. The probability of acquiring invasive listeriosis was highest for individuals with a solid organ transplant, with a median risk of 11 000 per million servings when the salad had been stored at 15 °C, compared with only 1 per million servings when the salad had been stored at 8 °C. For healthy adults

and the elderly, the risk of acquiring listeriosis was 28 and 530 per million servings, respectively, when the salad had been stored at 15 °C, while it was <1 per million servings for both these groups when the salad had been stored at 8 °C. However, there were large confidence intervals for these risk values, which illustrates the great uncertainty. For example, for the elderly with a median risk of 11 000 per million servings (stored at 15 °C), the 90% confidence interval for the infection risk was 4-220 000 per million servings.

To reduce the uncertainties associated with absolute risk estimation, relative risk was also estimated. For all population subgroups, the risk of listeriosis was 16-fold higher on consuming a mixed-ingredient salad stored at 8 °C at the end of shelf-life, or 200 000-fold higher when the salad was stored at 15 °C, compared with consumption on the day of inoculation. Hence, preventing temperature abuse during storage is of critical importance in mitigating the risk of foodborne listeriosis from these mixed-ingredient salads.

4.2.3 Risk modelling of *Escherichia coli* O157:H7 *gfp*+

The risk of *E. coli* O157:H7 infection, illustrated by the findings of *E. coli* O157:H7 *gfp*+, was high even at the initial level of 50-100 CFU/g, since only a few bacterial cells (<100) may cause human infection and illness (Teunis *et al.*, 2004). The lowest risk of *E. coli* O157:H7 infection after consuming one serving of baby spinach (30 g) was approximately 60% (600 000 per million servings) for baby spinach that had been stored at 8 °C for seven days, with slightly reduced numbers of *E. coli* O157:H7 *gfp*+ compared with the inoculated level. When baby spinach had been stored at 15 °C for seven days with slightly increased numbers of *E. coli* O157:H7 *gfp*+, the risk of acquiring infection was close to 100%. Since the weight of one serving of mixed-ingredient salad (300 g) was 10-fold that of one serving of baby spinach (30 g), the risk of *E. coli* O157:H7 infection when consuming one mixed-ingredient salad (300 g) initially contaminated with 50-100 CFU/g was 100% already on day 0, and also after storage at 8 or 15 C for seven days. Calculations of relative risk were not illustrative in these models, since there were similar risks for all conditions, due to the low number of *E. coli* O157:H7 cells that can cause disease. Thus, *E. coli* O157:H7 bacteria only need to survive in a food product to pose a risk of disease in humans, *i.e.* they do not need to multiply. Moreover, any cross-contamination during processing and preparation would present a human health risk. Therefore, it is of the utmost importance to prevent contamination of food products such as leafy vegetables by this pathogen at all stages from farm to fork.

4.3 Microbiota in ready-to-eat salads

4.3.1 Total viable aerobic counts and considerations on organoleptic alterations

In Paper II, there was no evaluation of organoleptic alterations, which may have been significant in samples stored under temperature abuse, reaching total viable aerobic counts of 9-10 log CFU/g (Figure 10). High viable aerobic counts (9.1 log CFU/g) were also observed in a similar previous study in which lettuce mixed with cooked chicken was stored at 14 °C, but after three days instead of seven (Bovo *et al.*, 2015). For the spinach leaves that had been stored at 15 °C for seven days in Paper II, a few leaves showed signs of decay with soft edges (Figure 11a). In addition, there was a change in the colour of the leaves from dark green to yellow in some cases, probably due to gradual loss of chlorophyll, as previously observed after storage of leafy vegetables at high temperatures (Kou *et al.*, 2014). However, for the samples stored for three days, which is the recommended shelf-life of mixed-ingredient salads, there were no changes in visual appearance (Figure 11b). In another study (Luo *et al.*, 2009), it was shown that spinach leaves that had been stored at 12 °C for six days had fully acceptable visual quality, despite *E. coli* O157:H7 being present at a level of approximately 5.5 log CFU/g. Thus, since growth of pathogens in leafy vegetables may emerge before the spoilage flora affect sensory attributes, consumer acceptance based on visual appearance and flavour may not be sufficient to ensure food safety.

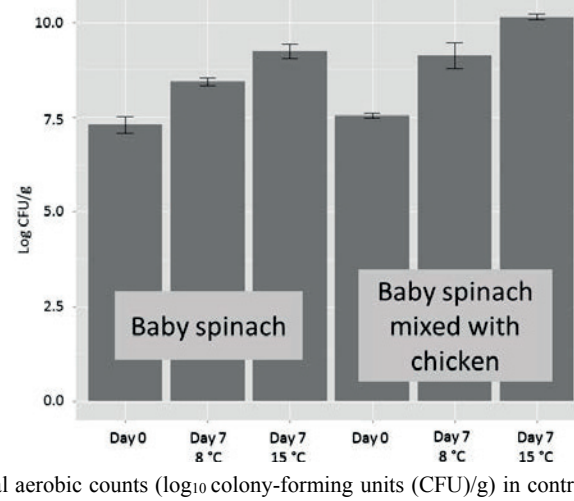


Figure 10. Total aerobic counts (log_{10} colony-forming units (CFU)/g) in control samples of baby spinach and baby spinach mixed with chicken before (day 0) and after (day 7) storage. The vertical bars represent standard error.

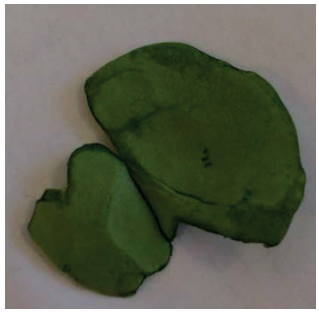
a)  b) 

Figure 11. (a) Baby spinach showing signs of decay after seven days of storage at 15 °C. (b) Baby spinach after incubation at 15 °C for three days (photos by author).

4.3.2 Exploration of microbiota based on Illumina 16S rRNA amplicon sequencing

Microbiota of RTE salads during storage

An average of 58 (range 18-90) Operational Taxonomic Units (OTUs) were detected in each sample, which can be interpreted as the approximate number of bacterial species to which a consumer will most likely be exposed on consuming RTE baby spinach or mixed-ingredient salad. Across all samples, a total of 190 OTUs were detected, representing four different bacterial phyla. Earlier work has shown that bacterial diversity in the phyllosphere of leafy vegetable samples collected from the field is greater than the diversity after packaging and storage (Lopez-Velasco *et al.*, 2011; Handschur *et al.*, 2005). For example, storage at 4 °C for only one day has been found to reduce the number of phyla from 11 to five (Lopez-Velasco *et al.*, 2011). The lower number of phyla detected in Paper III was expected, since the baby spinach had already been processed (*e.g.* washed in potable water), packaged and transported from the packaging facility to a retail store before being analysed. The composition of bacterial communities changed during storage, with Pseudomonales (mainly *Pseudomonas* spp.) being the dominant order across all samples and with the highest proportion (approx. 70%) occurring in mixed-ingredient salad that had been stored at 8 °C for seven days (Figure 12). The proportion of Enterobacteriales (mainly *Erwinia* and unclassified *Enterobacteriaceae*) was initially small (<5%) but increased to approximately 10-20% of the total community after seven days of incubation. *Pseudomonas* spp. have been reported previously to be among the dominant bacterial populations in RTE spinach and mixed vegetable salads during cold storage (Lopez-Velasco *et al.*, 2011; Rudi *et al.*, 2002; Babic *et al.*, 1996). *Enterobacteriaceae* have also been shown to be important members of the

phyllosphere community, particularly under temperature abuse (Lopez-Velasco *et al.*, 2011; Rudi *et al.*, 2002). In Paper III, Enterobacteriales was mostly represented by *Enterobacteriaceae,* including *Erwinia* spp. and unclassified *Enterobacteriaceae*. The unclassified *Enterobacteriaceae* may be represented by several genera with different features, including human pathogens of faecal origin. Species of *Pseudomonas* and *Erwinia* are widely distributed in the environment and some species are potential plant pathogens (Palleroni, 2015) or pectinolytic species important for spoilage of vegetables (Adams & Moss, 2005).

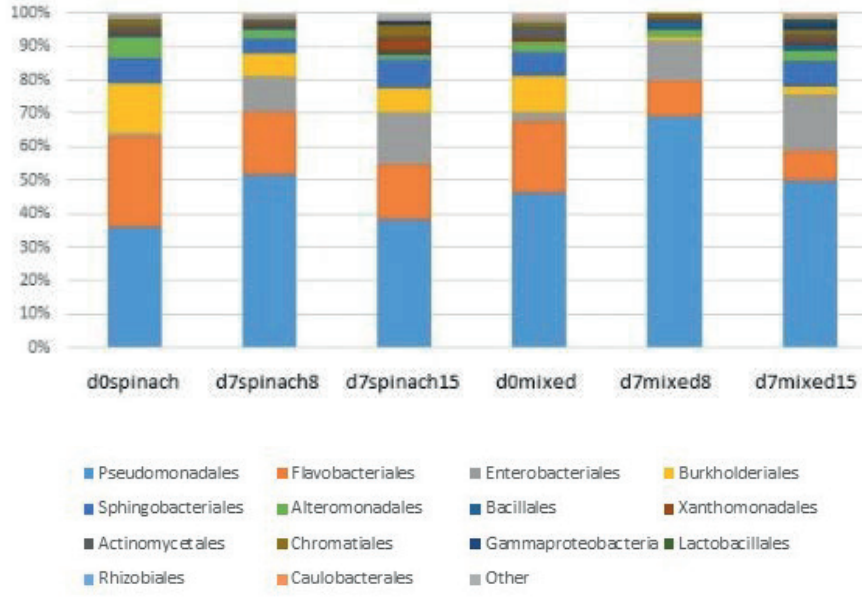
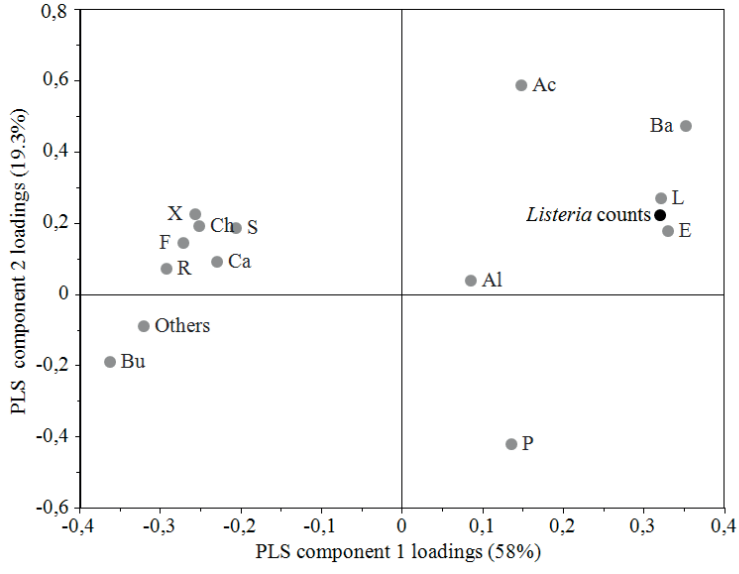


Figure 12. Overview of abundance of different orders of bacteria in control samples of baby spinach (spinach) and mixed-ingredient salad (mixed) before (d0) and after (d7) storage at 8 °C (8) or 15 °C (15). Gammaproteobacteria includes bacteria in this class that were not classified at order level.

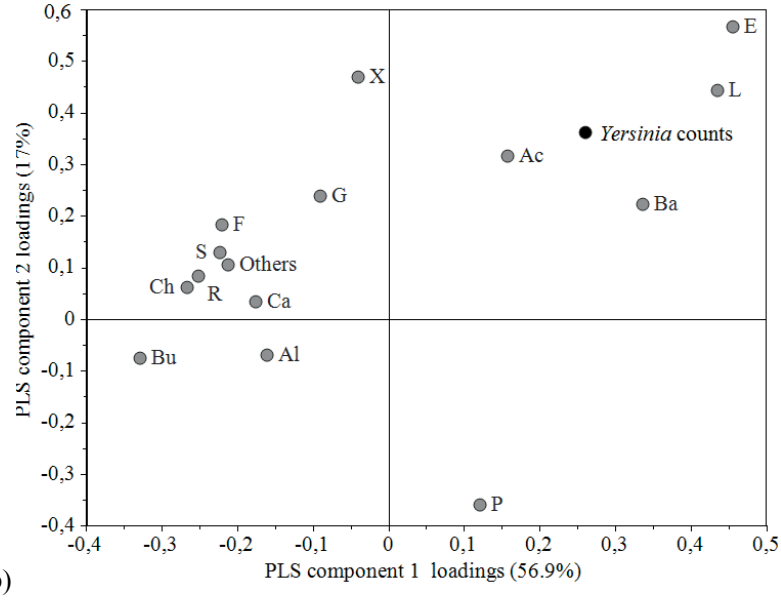
Associations between microbiota and foodborne pathogen contaminants

Based on the partial least squares (PLS) models (Figure 13 a, b, c), there were positive correlations between viable counts of inoculated strains on day 7 and the parallel abundances of the orders Bacillales, Lactobacillales and Enterobacteriales. This was generally confirmed by Spearman rank sum correlations. Some of these correlations were not surprising, since *L. monocytogenes* is affiliated to Bacillales while *E. coli* O157:H7 gfp+ and pathogenic *Y. enterocolitica* are affiliated to Enterobacteriales. Reasons for these correlations may be that inoculants and bacteria included in these orders are

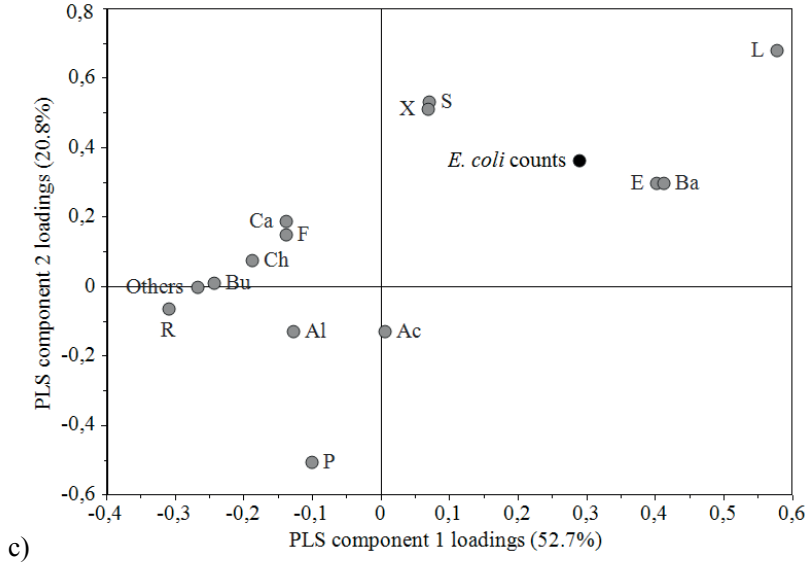
stimulated by the same conditions or possibly that they facilitate each other's growth due to synergistic utilisation of nutrients by yet unknown mechanisms.



a)



b)



c)

Figure 13. Loadings of the partial least squares (PLS) regression analysis of order taxonomical composition prediction of viable counts of inoculated strains. The graph shows how Y-variables representing viable counts of a) *Listeria monocytogenes*, b) pathogenic *Yersinia enterocolitica* and c) *Escherichia coli* O157:H7 *gfp*+ correlated with X-variables representing orders as follows: P: Pseudomonadales, F: Flavobacteriales, E: Enterobacteriales, Bu: Burkholderiales, S: Sphingobacteriales, Al: Alteromonadales, Ba: Bacillales, X: Xanthomonadales, Ac: Actinomycetales, Ch: Chromatiales, L: Lactobacillales, R: Rhizobiales, Ca: Caulobacterales and Others. The plot can be read by drawing a line from the Y-variable through the origin and across the plot. X-variables situated near Y-variables are positively correlated to them and those situated on the opposite side are negatively correlated. The X-variables situated near each other are correlated.

Identification of inoculated strains among OTUs

The number of bacterial sequences obtained from each individual sample was on average 9960, but ranged from 2 to 58 680. Three samples had read counts <1000 and were excluded. The other samples (n=51) were normalised, *i.e.* randomly subsampled to 1000 sequences in each sample, to avoid biases from uneven sampling efforts. This sequencing depth theoretically enables detection of populations making up more than 0.1% of the total community. The inoculated *L. monocytogenes* strain was identified amongst OTUs when present in viable concentrations of 9 log CFU/g. Since total aerobic counts (based on culture methods) were approximately 10 log CFU/g in corresponding samples, *L. monocytogenes* represented 10% of total viable counts in those samples. *Escherichia coli* O157:H7 *gfp*+ and pathogenic *Y. enterocolitica* were present in viable concentrations of approximately 7 log CFU/g in some samples, which

accounted for approximately 0.1% of total counts in those samples. Nevertheless, they could not be identified amongst OTUs. Therefore, in salads where concentrations of indigenous microorganisms are likely to be high, pathogens present in viable numbers of 7 log CFU/g will not readily be robustly identified in OTU tables from Illumina sequencing as performed in this thesis.

Based on the molecular method, the OTU that represented inoculated *L. monocytogenes* represented 2.2% of the total reads for these specific samples, compared with 10% representation based on cultural methods. Consequently, this indicates that the molecular method detects 4-5 times more bacteria present in the background microbiota of the sample than total viable counts detected by the culturing method. The difference may partly be due to accumulation of damaged or dead microbial cells during the incubation and/or presence of viable, but not culturable bacteria.

5 Conclusions

- Almost 1 in 10 RTE mixed-ingredient salads sampled at Swedish retail outlets was found to be contaminated with foodborne pathogens or presumptive pathogens. *Listeria monocytogenes* was isolated from two of 141 samples analysed. The other findings involved detection of virulence genes present in pathogenic *Y. enterocolitica* and STEC, but these could not be culturally confirmed.

- Mixing baby spinach with a protein source such as cooked chicken (mixed-ingredient salad) strongly influenced growth of inoculated *L. monocytogenes*, pathogenic *Y. enterocolitica* and *E. coli* O157:H7 *gfp*+, especially when stored under temperature abuse (15 °C). Growth of pathogens in leafy vegetables emerged before the spoilage flora affected visual appearance, and thus a fresh appearance does not ensure food safety. Storage of mixed-ingredient salads at 8 °C for three days complies with current recommendations in Sweden, but since these conditions did not prevent growth of *L. monocytogenes* they are not appropriate for this product and need to be adjusted.

- The estimated risk of *E. coli* O157:H7 infection, illustrated by the findings of *E. coli* O157:H7 *gfp*+, was high even at initial levels in servings of RTE baby spinach and mixed-ingredient salads. Thus *E. coli* O157:H7 did not need to multiply, but only to survive, in baby spinach or mixed-ingredient salad in order to pose a high risk of disease in humans.

- The estimated risk of listeriosis was 16-fold higher on consuming a mixed-ingredient salad stored at 8 °C at the end of shelf-life, or 200 000-fold higher when the salad was stored at 15 °C, compared with consumption on the day of inoculation. Preventing temperature abuse

during storage is therefore of crucial importance to mitigate the risk of foodborne listeriosis from mixed-ingredient salads.

- The composition of bacterial communities changed during storage of RTE baby spinach and mixed-ingredient salads, but with Pseudomonadales as the most abundant order across all samples. There were positive correlations between viable counts of *L. monocytogenes*, pathogenic *Y. enterocolitica* and *E. coli* O157:H7 *gfp*+ and abundance of Lactobacillales, Enterobacteriales and Bacillales, respectively. Thus inoculants and bacteria in these orders may be stimulated by the same conditions or possibly facilitate each other's growth by some unknown synergistic mechanisms. Although pathogens were present at high viable counts in some samples, they were only detected in the community-wide 16S rRNA amplicon dataset in samples where the pathogen represented approximately 10% of total viable counts.

6 Future perspectives

While many people are willing to adopt a healthier plant-based diet, others will need to be induced to do so due to the global situation with an urgent need to reduce greenhouse gas emissions. Sooner or later, the global human population will need to reduce its meat consumption and increase its consumption of vegetables. This will act as a catalyst for the development of new plant-based products in the future. The demand for convenience foods among busy consumers will continue, and more products based on fruit and vegetables and composite foods that are prepared and RTE will become available. Consumers need to be protected from the risks associated with these products. For example, one single event of STEC contamination in a field of growing leafy vegetables poses a human health risk. Therefore, improved guidance and for some critical events mandatory regulations are needed for these products from farm to fork to prevent foodborne disease.

While growers need to understand the potential risk of pathogens being present in the field environment, food handlers and consumers need to understand the importance of hygienic handling and cold storage of the final product. Information campaigns may be one way to educate consumers and there are some good examples of consumer guidance on the websites of governments or associated organisations in some countries, for example Canada (Government, 2015) and the U.S. (Fight, 2016b), but not yet in Sweden.

For growers, the quality of irrigation water needs to be regulated to reduce the risk of contamination. Water decontamination equipment on irrigation system end-points could also be one way to prevent contamination. However, contamination from wildlife can never be completely avoided in a field environment, and thus the development of new growing systems in a controlled environment may be another way forward. In addition, innovative strategies based on the use of biocontrol agents, for example introduction of protective bacteria in wash water, may be developed to increase food safety of RTE leafy vegetables (Siroli *et al.*, 2015). While 16S rRNA amplicon sequencing is not a

useful tool for detecting pathogens, it may be helpful in studying potential candidates for inhibiting human pathogens on leafy vegetables, since these protective bacteria must be able to thrive in the leaf microbiota under intended storage conditions.

In Sweden, the conventional refrigerator temperature of maximum 8 °C should be lowered. A cold chain with a temperature of maximum 4 °C would reduce both food waste and the risk of pathogen growth during storage (TemaNord, 2016). However, leafy vegetables that have not been washed or shredded do not need to be stored at refrigerator temperature according to current recommendations in Sweden. They may thus be presented in bags at room temperature, which will increase the risk of pathogen growth. There is a risk of some consumers not distinguishing between RTE leafy vegetables and unwashed, non-RTE leafy vegetables, which often have a similar appearance when presented in bags. Therefore, the safety of these products should be evaluated.

There have been improvements in surveillance and outbreak detection, and in EU a new Joint EFSA-ECDC Molecular Typing Database for *Salmonella, L. monocytogenes* and pathogenic *E. coli* with isolates from humans, food, animals and feed will soon be introduced. However, there are still many challenges when tracing the source of an outbreak. Methods to enable isolation and thus typing of *e.g.* STEC or pathogenic *Y. enterocolitica* still need to be improved. The present situation is that markers of pathogenic strains can be detected in food samples, but the findings are rarely culturally verified.

7 Populärvetenskaplig sammanfattning

Att äta hälsosamt och miljömedvetet är en trend i dagens samhälle och vi konsumerar alltmer grönsaker. Utbudet av ”ätfärdiga” bladgrönsaker som skurits och sköljts och är färdiga att serveras ökar. Det finns också ett stort utbud ätfärdiga matsallader där bladgrönsaker blandats med andra ingredienser, till exempel kyckling och pasta, för att utgöra hälsosamma och lättillgängliga måltider för konsumenterna. Parallellt med denna utveckling har det skett en ökning av livsmedelsburen smitta som kopplats till bladgrönsaker.

Smittämnen kan tillföras vid flera av stegen i produktionen av ätfärdig sallad, till exempel via bevattningsvatten under odlingen av bladgrönsaker eller under den manuella hanteringen av de olika salladsingredienserna vid tillredningen av en matsallad. För ätfärdiga sallader finns inget steg i produktionskedjan som kan eliminera smittämnen som eventuellt hamnat i produkten, till skillnad från till exempel kött där avdödning av smittämnen sker vid tillagning. För att salladsprodukter ska vara säkra för konsumenterna måste de produceras under goda hygienförhållanden samt förvaras vid en korrekt temperatur.

I denna avhandling har livsmedelssäkerheten för salladsprodukter studerats. I den första studien analyserades ätfärdiga matsallader från butik och i ungefär var tionde sallad fanns indikationer på förekomst av bakterier som kan orsaka livsmedelsburen sjukdom hos människa. I två av 141 undersökta sallader hittades bakterien *Listeria monocytogenes* som kan leda till listerios, en sällsynt men allvarlig sjukdom som framför allt drabbar personer med nedsatt immunförsvar, gravida eller äldre.

I den andra studien undersöktes smittämnens förmåga att föröka sig i ätfärdig sallad. De tre smittämnen som undersöktes (*Listeria monocytogenes*, patogen *Yersinia enterocolitica* och *Escherichia coli* O157:H7) kunde nå mycket höga nivåer i matsallad som förvarats vid en felaktig temperatur (15 °C) under hållbarhetstiden. För en matsallad som förvarats i 15 °C bedömdes risken för listerios öka hundratusenfalt jämfört med om matsalladen ätits direkt efter tillblandning. Både handlare och konsumenter bör därför informeras om vikten

av korrekt kylförvaring av matsallader för att förhindra tillväxt av smittsamma bakterier och därmed livsmedelsburen sjukdom. *Listeria monocytogenes* kunde växa i blandsallad även vid rekommenderad förvaringstemperatur (8 °C), vilket indikerar att denna temperatur bör sänkas.

I den tredje studien användes en DNA-baserad metod för att ge en överskådlig bild av de bakterier som fanns i prover av babyspenat och matsallad. I dessa produkter finns normalt ett mycket stort antal bakterier, uppemot 100 miljoner bakterier per gram, som vanligtvis är ofarliga för människor men som har varit svåra att studera med hjälp av traditionella metoder baserade på odling. Vissa av de undersökta proverna innehöll förutom den normala bakteriefloran även tillsatta smittämnen, liksom i föregående studie. I dessa prover sågs samspel mellan de tillsatta smittämnena och vissa bakteriegrupper i den normala salladsfloran. Denna kunskap kan vara en pusselbit i fortsatta studier med målet att hitta bakteriegrupper som kan verka skyddande mot tillväxt av oönskade smittämnen i sallad och på så sätt minska risken för livsmedelsburen sjukdom. Den DNA-baserade metoden är relativt ny och kan endast se lågupplösta mönster i bakteriefloran. Även då de tillsatta bakterierna fanns i ett mycket stort antal, tillräckligt för att kunna orsaka sjukdom hos människa, gick de inte att upptäcka med hjälp av denna metod. Först då de tillsatta bakterierna utgjorde 1/10 av odlingsbara bakterier i ett prov, kunde de identifieras.

Sammantaget visar den här avhandlingen att matsallad är en riskprodukt. Goda hygienrutiner under produktionen av samtliga ingredienser och under tillredningen samt en korrekt kylförvaring är av största vikt för att minska risken för livsmedelsburen sjukdom orsakad av matsallad.

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