POST-HARVEST INTERVENTIONS AND FOOD SAFETY OF LEAFY GREEN VEGETABLES

DISSERTATION

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By

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Abstract

Foodborne illness outbreaks associated with the consumption of leafy green vegetables are a growing public health concern worldwide. While there has been great progress in using practical and cost-effective interventions to reduce the risk of contaminations and prevalence of pathogens on farm, effective post-harvest interventions to remove field acquired contamination are still lacking. The body of literature related to microbial hazards in leafy green vegetables has accumulated since 1990, offering often contradictory information on the efficacy of food safety interventions. In this work, I identified, characterized, and assessed the quality of available research on prevalence, risk factors, and interventions for 16 microbial hazards in leafy green vegetables. Systematic literature review, a replicable two-level relevance screening, and a two-phase quality assessment and data extraction procedure were performed by two independent reviewers following general principles of systematic review methodology. A lack of well designed, executed, and reported prevalence studies investigating the efficacy of intervention(s) under real-life conditions was observed. Additional identified knowledge gaps and research areas included equipment sanitation and cross-contamination potential, survival of pathogens in organic leafy greens, and the lack post-harvest intervention studies applicable to the developing regions.
Several identified knowledge gaps were further addressed. The concentration of coliforms and generic *Escherichia coli* and prevalence of *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp. and *Listeria monocytogenes* on two spinach types before and after minimal processing under commercial conditions was investigated. A total of 1356 spinach samples were collected daily in two processing plants over a period of 14 months. Average total coliform counts increased significantly after processing. The proportion of coliform positive samples increased from 53% before to 79% after minimal processing. Generic *E. coli* prevalence was 8.9% (mean 1.81 ± 0.14 log CFU/g) with no difference after processing. *Salmonella* spp. and *L. monocytogenes* were isolated from 0.4% and 0.7% of samples, respectively. The contribution of each commercial processing step to generic *E. coli*, coliform, and total aerobic bacterial load on fresh-cut lettuce and food contact surfaces in a processing plant were further quantified. Plausible bacterial populations on fresh-cut, minimally processed lettuce using T-RFLP analysis were also identified. Washing initially decreased coliforms on lettuce but microbial populations increased to the pre-washed levels during subsequent steps. Peak contamination on food contact surfaces was detected after two hours of processing, and then declined after 4-6h of processing without intermediate sanitation interventions. No association between coliform increase on lettuce and contact-surfaces was found. Removal of outer leaves was the single most effective step of large-scale minimal processing. T-RFLP analysis identified representatives of 12 phyla/classes with the bacterial population profile from minimally processed lettuce dominated by Gammaproteobacteria and Bacteroidetes.
The ability of *E. coli* O157:H7 to survive in processed packed organic and conventional baby spinach under household storage conditions for the duration of shelf life was further compared. Epiphytic bacteria present and the effect of leaf size on survival of *E. coli* O157:H7 were assessed. *E. coli* O157:H7 survived equally well on both spinach types. Organic spinach had overall smaller leaves that supported higher counts of *E. coli* O157.

Finally, existing data pertaining to post-harvest interventions was compiled and synthesized. The options for post-harvest risk reduction were summarized and the existing information was translated to vegetable production conditions in developing countries. The applicability of multiple-barrier approach in developing regions was discussed.

This work fills several critical gaps in knowledge. The scoping study provides a model for similar studies that can identify gaps in knowledge and thus, target limited resources and guide policy development. Information gained in studies of minimal processing can be used to develop more effective measures appropriate to control food safety risks in commercial settings. This work has a direct impact on pre-harvest food safety enhancement for leafy green vegetables.
To Alex, Daniel & Anna
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Fields of Study

Major Field: Food Science and Technology
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Chapter 1- Food Safety of Fresh Produce: Public Health Challenge

Justification and Rationale

Fresh fruits and vegetables are an indispensible part of a healthy and balanced diet (Carter, Gray, Troughton, Khunti, & Davies, 2010; Hamer & Chida, 2007). Consumer demand for fresh and minimally processed vegetables has steadily increased in the past three decade due to the healthier diet trends and the convenience of modern packaging. Per capita fresh vegetable consumption increased from around 141.3 lb in 1990 to 175 lb in 2006 the US (Figure 1.1) (Lucier & Glaser, 2010). However, the consumption in both US and Canada has declined in the last five years by an average 8.8-11 lb per capita. (Lucier & Glaser, 2010; Statistics Canada, 2009). Despite numerous intensive health education programs (Centers for Disease Control and Prevention (CDC) and Produce for Better Health Foundation (PBH), ) a consumer’s choice to include fresh vegetables in the daily diet is currently heavily influenced by the awareness of increasing numbers of reported outbreaks of foodborne disease (Harris Interactive, 2007).

Outbreaks associated with consumption of fresh fruits and vegetables

The risk of foodborne illnesses from consumption of fresh fruit and vegetables is a top priority public health concern. Several recent outbreaks associated with fresh
vegetables caused large number of illnesses and affected wide geographical areas. An *E. coli* O157:H7 outbreak linked to fresh spinach in 2006 caused 205 illnesses, resulting in 141 hospitalizations, 31 cases with kidney failure (Haemolytic Uremic Syndrome –HUS), and three deaths in the US and Canada (Center for Disease Control and Prevention (CDC), 2008). In 2008, a *Salmonella* Saint Paul outbreak from Jalapeño peppers affected 43 states, caused 1,442 illnesses, and was originally blamed on tomatoes ((Center for Disease Control and Prevention (CDC), 2008). In 2011, an ongoing *E. coli* O104:H4 outbreak in Europe linked to fresh produce affected 3,908 individuals in total, including 765 cases with HUS and 41 fatalities in Germany and 12 other European Union countries. In addition to resulting in a major public health burden, all of these outbreaks lead to significant losses for the fresh produce industry. The spinach outbreak in 2006 cost local growers in California nearly $6 million in lost revenue (Chediak 2006). Compensation of $306.2 million was offered to European vegetable growers by the European Commission to cover some losses incurred from the outbreak of *E. coli* O104:H4 (Chelsom-Pill & Hallam, 2011). In the US, aggregate cost of illnesses attributed to fresh produce consumption is estimated to be $39 billion each year (Scharff, 2010).

Between 1990 and 2006, the Center for Science in Public Interest (CSPI) identified a total of 768 outbreaks associated with fresh produce, with 35,060 cases of illness. Fresh produce became a single commodity responsible for the largest number of cases, constituting 21% of reported cases (Smith DeWaal, Tian, & Plunkett, 2009). However, comprehensive reports describing the characteristics of these outbreaks are currently lacking. During our systematic literature review for this dissertation and
through collaboration with the Public Health Agency of Canada, scoping studies reporting outbreaks linked to fresh produce from 1998-2008 were performed. Extracted data were merged with the data from two major North American databases: OutbreakAlert! from the CSPI and the Microbial Food Safety Database (MFSD) from Public Health Agency of Canada. In order to provide up-to-date epidemiological information, a brief summary of the identified outbreaks is presented here.

A total of 643 outbreaks of foodborne illness linked to consumption of fresh vegetables were recorded in the period of 1998 to 2008 in North America. Of those, 613 occurred in the USA, 24 in Canada, and 6 affected populations on both sides of the border. The total number of cases in these outbreaks was 31,027.

In the period of 1998 to 2008, the number of outbreaks associated with leafy green vegetables has been steadily high (Figure 1.2). Leafy greens, not including leafy green herbs, were responsible for a little under half (44%, n=283) of all outbreaks linked to fresh produce and 32% of the total number of cases. While this high proportion of outbreaks linked to consumption of leafy greens can be to some extent explained by consumption volume (Lucier & Glaser, 2010; K. Warriner, Huber, Namvar, Fan, & Dunfield, 2009b), certain pathogens and pathogen-produce combinations caused outbreaks more frequently. For instance, *Norovirus* caused 48% (n=295) of all outbreaks, accounting for 11,435 cases in total. Of these, 188 outbreaks of *Norovirus* were linked to leafy greens, making 6,448 people sick over 10 years. Similarly, 65% (n=42) of all *E. coli* outbreaks (n=65) were associated with consumption of leafy green vegetables yielding 1,483 cases of illness. *Salmonella* spp. accounted for 136 outbreaks; however 8,601 cases
of salmonellosis were linked to a variety of produce including tomatoes, alfalfa, melons, leafy greens, and other produce (Table 1.1). Tomatoes are most frequently contaminated with *Salmonella* spp. (n=22, 69% of all tomato linked outbreaks) and *Norovirus* (n=10; 22%). No outbreaks from *Listeria monocytogenes* linked to fresh produce in the past decade were detected, while cabbage was implicated in two reported outbreaks over ten years caused by viruses (Table 1.1).

Although improvements have been made over the past decade to improve traceability, the ability to track contaminated fresh fruits and vegetable back to the source is still lacking (K. Warriner et al., 2009b). Traceback of outbreaks linked to fresh produce to the source presents challenges (Guzevich & Salsbury, 2000) and often the actual source of contamination remains a speculation. For this reason, and the fact that other epidemiological information from outbreak databases is missing, it is only possible to draw inferences based on a very small portion of outbreaks for which the relevant data exist. For instance, the country of origin information was mostly unknown. This information was available for only 37 out of 643 outbreaks, 12 of which fresh produce originated from the US. Contamination sources were identified for only 50% of these 37 outbreaks (2 *Salmonella* outbreaks were linked to contaminated alfalfa seed; 2 *Salmonella* outbreaks in tomato were linked to on-farm contamination, and two other *Salmonella* outbreaks in tomatoes were linked to failed hygiene practices). In five out of ten outbreaks traced back to produce from Mexico, contaminated water (ice or irrigation) was identified as the source; one outbreak was traced back to processing and three were contaminated by human handling. In two outbreaks of *Salmonella* spp., the implicated
mangos were from Brazil in one case and Peru in another. Both outbreaks were traced back to contaminated water in hot water treatment. Two *E. coli* O157 and one *Salmonella* spp. outbreaks were linked to contaminated alfalfa seed originating from Australia.

Information on organic and conventional produce was available for only 42 outbreaks where the data source were peer-reviewed publications. All 42 of the outbreaks were associated with conventional fresh produce. Similarly, whether the produce was grown in the field or in the greenhouse was the information available for only 43 outbreaks, all of which were linked to field grown produce.

Reported outbreaks of foodborne disease from fresh produce most often occurred in the restaurant. Over one half of 643 outbreaks (n=353) were linked to restaurants. Eighty-two outbreaks occurred at home, 72 at private gatherings, 29 in schools or day cares, 24 at catered events, 15 at retirement or senior homes, 2 from street-vendors, 2 from a military base or prison, 1 from the hospital, and 63 from other locations. Private gatherings were captured mostly by the OutbreakAlert Database (n=67). One half of the private gathering outbreaks (n=42) were from *Norovirus* and 24 of those were from *Norovirus*-leafy green combination. The data on sources of contamination are missing.

For outbreaks that occurred at home, source of contamination data were available for 21 outbreaks. None of these outbreaks were captured by systematic review of published outbreak reports. For the ones with a known source, unspecified on-farm contamination sources were identified as the source in six outbreaks, contaminated seed in five, contaminated water in four, and processing in three outbreaks. Lack of good
agricultural practices was linked to one outbreak and one outbreak was linked to wild bird contamination in the field.

Although the actual sources are unidentified in most of the outbreaks, available data highlight pre-harvest sources and minimal processing as main sources of contamination and the lack of effective measures to control contamination throughout fresh produce production chain is evident. At the same time, the reviewed outbreak data highlight the insufficient baseline knowledge of microbial safety of organically and greenhouse grown vegetables.

Pre-harvest contamination of fresh vegetables and control measures

The current understanding is that the plants, including fresh vegetables, are not natural hosts for enteric pathogens (Jacques, Kinkel, & Morris, 1995; Leveau & Lindow, 2001). Enteric bacteria, viruses, and protozoa are introduced into vegetable production systems as contamination. Fresh vegetables can become contaminated at any point in the farm-to-fork continuum, directly from animals (Franz, Semenov, & Van Bruggen, A. H. C., 2009; Mukherjee, Speh, Dyck, & Diez-Gonzalez, 2004)(Mukherjee et al., 2004; Mukherjee et al., 2006); Mukherjee et al., 2006) via a vector such as irrigation water or soil amended with manure (Bardet, 2007; Islam et al., 2004), or cross-contamination including on-farm and processing activities, in retail facilities or the consumer’s home.

Exposed to the environment in open fields, fresh vegetables and fruits are subject to multiple potential sources of pre-harvest contamination. While exact sources of
vegetable contamination in the field remain unclear, manure and soil amendments, contaminated water, and wildlife are likely contributors.

Food animals are recognized as reservoirs of human enteric pathogens (Beuchat, 2006; Franz, van Diepeningen, de Vos, & van Bruggen, 2005). These pathogens are shed in their feces, which may come in contact with vegetables and serve as a source of contamination. Although animal manure is an important fertilizer, particularly in the context of sustainable agriculture, the application of manure or biosolids to growing crops is a direct route of vegetable contamination (Franz, Semenov, & van Bruggen, 2008; Girardin et al., 2005; Ingham et al., 2005). Additionally, when soil is fertilized with animal manure or biosolids or farm and sewage effluents are involuntarily introduced (run offs), the contamination of vegetables largely depends on the ability of the pathogens to survive in the environment and on crops (Semenov, van Overbeek, & van Bruggen, Ariena H. C., 2009). In general, enteric pathogens can persist in manure and soil for extended periods of time (Kupriyanov, Kunenkova, Van Bruggen, A. H. C., & Semenov, 2009) depending upon temperature, humidity, cell concentration, and other factors (Girardin et al., 2005; Semenov et al., 2009). A broad range of adaptation mechanisms to a non-host environment has been reported in literature (Mootian, Wu, & Matthews, 2009; Semenov et al., 2009; Wei, Jin, Sims, & Kniel, 2010) conveying a variety of survival abilities. Different strains of Salmonella spp. and E. coli O157 show a range of responses to environmental stresses (Girardin et al., 2005; K. Warriner et al., 2009b), allowing them to persist in the environment different lengths of time (Semenov et al., 2009; Winfield & Groisman, 2003). Numbers of E. coli O157:H7 significantly declined but were still
detectable from both non-composted manure and amended soil after 17 weeks (Ingham et al., 2005). In moist bovine manure, another *E. coli* O157:H7 strain survived 49 to 70 days, depending upon the conditions (Semenov et al., 2009). Baloda et al. (2001) reported that *Salmonella* Typhimurium in soil amended with pig manure was undetectable 21 days after application. Girardin et al. (2005) showed that *Listeria monocytogenes* declined in numbers but persisted over 90 days in inoculated soil. *Clostridium* spp. spores declined only 0.7 log CFU after 16 months of study.

Agricultural water is one of the most important vectors of vegetable crop contamination. Water is very susceptible to contamination from manure or sewage (K. Warriner et al., 2009b). Run off after rainfall can transfer pathogens from animal or human waste and introduce them to vegetable growing soil or edible parts of the crop. Transfer of enteric pathogens to the growing vegetables via irrigation water was shown for a number of human pathogens. Bardet et al. (2007) showed presence of *Listeria* spp, *Bacillus cereus*, *Clostridium perfringens* and *E. coli* in irrigation water. This study reported transfer to growing parsley leaves within one day (Bardet, 2007). The ability of *Salmonella* Typhimurium to transfer from irrigation water to parsley was demonstrated by (Lapidotand & Yaron, 2009)Lapidot and colleagues (2009), and the transferred bacteria persisted on mature parsley leaves for over three weeks.

Wildlife is recognized as an important vector for pathogenic bacteria in an agricultural setting (Ingham et al., 2004). Reintroduction of *E. coli* to agricultural plots by wildlife may have played an important role in extended *E. coli* persistence findings by Ingham et al. (2005). However literature on the role of wildlife in contamination of fresh
vegetables is scarce. Potential for transfer of *E. coli* O157:H7 from feral swine to spinach in the field has been reported (Jay, Cooley, Chao, Wiscomb, & Mandrell, 2007).

While contamination via wildlife may be difficult to control on a farm (Ingham et al., 2005), animal manure and sewage can be treated to control microbial contamination. Proper composting can be sufficient to inactivate human pathogens in manure if adequate temperature, holding time, oxygen flow, moisture, and pH are maintained (Alfano, Belli, Lustrato, & Ranalli, 2008; Contreras-Ramos, Alvarez-Bernal, Trujillo-Tapia, & Dendooven, 2004; Semenov et al., 2009; U. S. Environmental Protection Agency (EPA), 2006); however, the recommended composting parameters are still under debate (K. Warriner, Huber, Namvar, Fan, & Dunfield, 2009a) mostly due to variations in reported persistence of human pathogens in manure (Cote & Quessy, 2005).

The method of applying irrigation water, such as drip or trickle and subirrigation, can be chosen to prevent direct exposure of edible portions of vegetables (FDA, 1998). Various decontamination treatments including chlorination and filtration (Olayemi, 1997) are also shown to reduce the microbial load in irrigation water and to prevent contamination of produce. Other control measures such as exposure of produce to sunlight (4 h) and raising crops on beds were reported to control coliform counts (Minhas, Sharma, Yadav, & Joshi, 2006). However, the key intervention to reduce the risk of contamination via irrigation water is monitoring of irrigation water microbial quality (K. Warriner et al., 2009b). Detection of the sources of contamination is crucial for development of improved and more effective control measures.
Post-harvest contamination sources

In addition to pre-harvest contamination sources, the post-harvest production chain presents multiple opportunities for introduction of human pathogens to fresh vegetables. Multiple steps involved in minimal processing of leafy green vegetables (i.e. cutting, washing, packaging) may provide opportunities for bacterial introduction via cross-contamination from either wash water, equipment, and contact surfaces or from contaminated batches of product (Doyle & Erickson, 2008; Stafford et al., 2008). The ability of human pathogens to persist on lettuce through minimal processing has been previously shown (Aruscavage, Miller, Ivey, Lee, & LeJeune, 2008; Ibekwe, Grieve, Papiernik, & Yang, 2009). For instance, on chopped lettuce and whole spinach leaves E. coli O157:H7 persisted throughout processing and decreased by 1.5 log at 4 ºC but increased at abusive temperatures (25ºC and 32ºC) (Doering, Harrison, Morrow, Hurst, & Kerr, 2009). E. coli O157:H7 survived sanitation steps after cross-contamination on fresh-cut lettuce (Lopez-Galvez, Gil, Truchado, Selma, & Allende, 2010). Procedures involved in minimal processing vary for each produce type and often involve fresh-cutting, washing, drying, and packing. In leafy greens, washing is primarily used for soil removal and does not always decrease the contamination on leafy green products.

Microbial interactions on the surface of fresh vegetables

The leaf surface, or phyllosphere, of leafy greens is inhabited by a variety of epiphytic microorganisms (Lindow & Brandl, 2003), of which bacteria are most abundant. Numbers of bacteria in phyllophere range from $10^5$ to $10^8$ CFU/g of leaf
Epiphytic bacteria may be plant pathogens or non-pathogenic organisms that are involved in a variety of processes that influence plant health, environment, and contribute to carbon and nitrogen cycles (Zhang et al., 2010). A number of epiphytic bacteria have been investigated as biocontrol agents or sources of bioactive components (Bashan & De-Bashan, 2002; Schreiber et al., 2005).

Interactions of epiphytic organisms with human pathogens on vegetable surfaces have been a topic of research that examines the microbial ecology associated with phyllosphere (Talias, 2010). Some fungi have been shown to enhance persistence of Salmonella on tomatoes through increasing the pH (Wade & Beuchat, 2003). Through competitive exclusion, Enterobacter asburiae has been shown to inhibit the growth of E. coli on Arabidopsis (Cooley, Miller, & Mandrell, 2003; Cooley, Chao, & Mandrell, 2006). The presence of Enterobacter and Bacillus spp. had a negative effect on the persistence of Salmonella on preharvest tomatoes (Shi, 2009). Many of the interactions that occur between human pathogens and epiphytic microbial communities on the surface of vegetables are just beginning to be elucidated (Critzer & Doyle, 2010). Microbial population profile changes in the presence of Salmonella spp. were studied for tomato (Shi et al., 2009). Profiles of epiphytic bacteria from the surface of spinach, rape, celery, broccoli, and cauliflower were reported by Zhang et al., 2010 (Zhang et al., 2010). Wetzel et al. 2010 described profile of microbial populations on basil leaves (Wetzel, Lee, Lee, & Binkley, 2010). The combination of antagonistic and synergistic interactions between enteric pathogens and epiphytic community may be altered during various...
minimal processing steps. To our knowledge, there have been no reports of bacterial community profiles of minimally processed lettuce.

Measures to control contamination of fresh vegetables

Failure of post-harvest interventions to remove field acquired contamination with human pathogens is evident (K. Warriner et al., 2009b). Post-harvest vegetable washing with sanitizers/disinfectants is the most commonly applied intervention and the most studied one. The effect of chlorine based sanitizers is reported in numerous studies (Casteel, Schmidt, & Sobsey, 2008; del Carmen Velazquez, Beatriz Barbini, Esther Escudero, Lucero Estrada, & Maria Stefanini de Guzman, Ana, 2009; Gragg & Brashears, 2010; Keskinen, Burke, & An nous, 2009; Kim, Kim, & Song, 2009; Lopez-Galvez et al., 2010; Lopez-Galvez, Gil et al., 2010; Takeuchi & Frank, 2001a; Takeuchi & Frank, 2001b). The reported reductions of human pathogens on leafy green vegetables with sanitizers vary greatly: from 0.6 log CFU/g (Koseki, Yoshida, Kamitani, & Itoh, 2003; Lee & Baek, 2008) up to over 5 log CFU/g (Lang, Harris, & Beuchat, 2004; Rodgers, Cash, Siddiq, & Ryser, 2004). The generally accepted reduction range for naturally contaminated vegetables is 1-2 log CFU/g (Doyle & Erickson, 2008). However, the reported reduction ranges from narrative literature reviews may not take into account the quality of included findings. Quantitative analysis that takes into account the differences between the studies is required to answer the question of overall effectiveness of vegetable wash sanitizers.
One of the purposes of minimal processing is to reduce the initial microbial load on vegetable surfaces (Gomez-Lopez, Ragaert, Debevere, & Devlieghere, 2008). However, different studies have demonstrated that washing and other sanitation treatments can enhance the growth rate of microorganisms. Total aerobic bacteria on chicory endive leaves disinfected with hydrogen peroxide were higher than on non-disinfected leaves during storage at 10 °C (Bennik et al., 1996). Similarly, psychrotrophic counts were higher on lettuce treated with warm, chlorinated water at refrigeration storage than lettuce left untreated (Li, Tajkarimi, & Osburn, 2008).

The overview of microbiological safety issues in fresh vegetable production indicates that significant knowledge gaps may exist associated with the risk of contamination, pathogen transmission, and control during minimal processing of leafy greens. Although a large number of research reports pertaining to these issues are available, numerous questions regarding food safety of leafy greens remain unanswered. To date it is not know 1) what is the overall effect of commercial minimal processing on contamination of packed leafy green vegetables, and 2) to what extent each step of processing contributes to the changes in contamination levels. In addition 3) bacterial community profile of minimally processed leafy greens is not well understood. Understanding how post harvest interventions alter contaminants and epiphytic bacterial community on leafy green vegetables is necessary to improve existing and identify novel strategies to mitigate the risk of foodborne illnesses linked to leafy greens and ultimately enhance food safety of these foods. In addition there is a need to understand the sources of microbial contamination of leafy green vegetables.
The focus of this dissertation was to provide answers to these questions by addressing the critical need to understand the effect of commercial minimal processing on bacterial contamination of leafy green vegetables.

Using and expending upon the approach of Risk Analysis, several complementary studies that address the changes that occur during minimal processing of leafy greens in the commercial processing scale, and at the molecular level in the laboratory setting were performed. The specific aims, working hypotheses and objectives are outlined below:

1. **Current research gaps related to microbial hazards in leafy green vegetables**

This research was based on the critical need to synthesize and determine the quality of existing evidence related to microbial hazards in leafy green vegetables, to identify the knowledge gaps and extract the data necessary for Risk Assessment in the field.

**Objectives:**

a) To conduct a scoping study of publicly available research that investigates prevalence, risk factors, and interventions for 16 microbial hazards in leafy green vegetables (Chapter 2).

b) To characterize the volume, main characteristics, basic aspects of methodological soundness and reporting; and main gaps and research needs in this area (Chapter 2).
c) To identify areas where there are currently sufficient research for future application of systematic review-meta-analysis methodology (chapter 2).

2. Changes in contamination levels during minimal processing of leafy green vegetables

Minimal processing as a Risk Management strategy to control foodborne pathogens in leafy green vegetables at the processing level was assessed by testing the working hypothesis that minimal processing reduces the counts of total aerobic bacteria and coliforms and prevalence of human pathogens in leafy green vegetables.

Objectives:

a) To determine the effect of a commercial minimal processing practice on the bacterial counts of coliforms and generic Escherichia coli (Chapter 3).

b) Determine the prevalence of foodborne pathogens (Escherichia coli O157:H7, Salmonella spp, Shigella spp. and Listeria monocytogenes) in minimally processed and unprocessed spinach in commercial setting (Chapter 3).

c) To quantify the contribution of each processing step to generic Escherichia. coli, coliform, and total aerobic bacterial counts on fresh-cut lettuce and food contact surfaces in a commercial processing plant (Chapter 4).

d) To identify minimal processing procedures associated with changes in microbial concentrations in a processing plant (Chapter 4).

e) To determine plausible bacterial population structure of fresh-cut, minimally processed lettuce (Chapter 4)
3. **Survival of pathogens in minimally processed leafy green vegetables produced by different farm management practice**

The working hypothesis tested was that the die-off rate of inculcated E. coli O157:H7 is higher in organic than in conventionally grown baby spinach.

**Objectives:**

a) To compare the ability E. coli O157:H7 to survive in retail organic minimally processed bagged baby spinach to conventionally grown spinach under refrigerated storage during 14 days of shelf life (Chapter 5)

b) To assess the effect of naturally present lactic acid bacteria on survival of E. coli O157:H7 (Chapter 5).

4. **Applicability of multiple-barrier approach to control and reduce microbial hazards in post-harvest chain of fresh vegetable production in developing countries**

The lack post-harvest intervention studies applicable to the developing regions was identified as one of the knowledge gaps in the field. The Risk Communication targeting developing countries was developed to address the need to enhance applicability of existing evidence to the culture and conditions in developing countries. Options for post-
harvest risk reduction were summarized and the existing information was translated to vegetable production conditions in developing countries.
Figure 1.1 Consumption of fresh fruits and vegetables 1998-2011.

Figure 1.2. Foodborne illness outbreaks linked to fresh produce in the USA and Canada from 1998-2008. Total number of outbreaks and those from *E. coli*, *Salmonella* spp., and *Norovirus* are shown. Data source: systematic literature review data merged with Outbreak Alert and MFSD databases.
Table 1.1. Human pathogens and produce involved in outbreaks associated with the consumption of fresh produce in Canada and the USA, 1998-2008

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<th>E. coli</th>
<th>S. anatum</th>
<th>G. lamblia</th>
<th>H. pylori</th>
<th>N. meningitides</th>
<th>S. flexneri</th>
<th>S. typhi</th>
<th>S. agalactiae</th>
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1 Subtotals for categories for leafy greens (n=281) and leafy green herbs (n=23) are not included in total sum (n=643)

2 Includes salsa, guacamole, pear, pineapple, garlic, lemon, grapefruit, mamey, blackberry, mushroom, and celery
References


Harris Interactive. (2007). Food safety warnings receive attention the public; but few report food-related illnesses to governing agencies or food providers. *WSJ Online/Harris Interactive Health-Care Poll, 6*, 1-5.


fertilized with noncomposted bovine manure: Garden-scale studies. *Applied and Environmental Microbiology, 70*(11), 6420-6427.


Abstract

A scoping study was conducted to identify all published prevalence, risk factor, and intervention research investigating 16 microbial hazards in leafy green vegetables and to evaluate the volume, main characteristics, basic aspects of methodological soundness and/or reporting, and the main knowledge gaps and research needs. Our study included a comprehensive literature search, a replicable two-level relevance screening (abstract and article levels), and a two-phase quality assessment and data extraction (article level). All steps were conducted by two independent reviewers following general principles of systematic review methodology. From the initial 7,961 citations, 657 articles were relevant, reporting one or more research themes: prevalence (314 studies), risk factor (472) and intervention research (269). These articles were published in 190 different scientific journals, 15% between 1990 and 2000, and the remaining 85% after year 2000. Sixty-five percent of studies were conducted in the USA, Canada, or Europe. Over 70% of all studies investigated lettuce. Collectively, four leafy greens (lettuce,
cabbage, spinach, and fresh leafy herbs) and microorganism (E. coli, Salmonella, Listeria and coliforms) combinations accounted for almost 80% of relevant studies. Forty-one percent of the research was conducted at the processing stage of production. Lack of reporting sufficient data and/or replicable laboratory protocols (first phase assessment) resulted in exclusion of 60% of relevant articles. In total, 231 papers were retained following second phase quality evaluation, and only 152 (20% of all relevant articles) met all ranking criteria. A lack of well designed, executed, and reported prevalence studies with sampled populations representative of the target populations and of experimental studies investigating the efficacy of intervention(s) under real-life conditions was observed. A limited number of articles investigating commonly accepted important risk factors, for example worker hygiene and health, equipment sanitation, and wildlife, were identified. We highlight research areas with the data potentially feasible for full systematic review-meta-analysis methodology and areas warranting additional investigation. The resulting information is necessary for the establishment of evidence-informed guidelines for food safety enhancement.

Introduction

Fresh fruits and vegetables are an important part of a healthy and nutritious diet (Carter, Gray, Troughton, Khunti, & Davies, 2010; Hamer & Chida, 2007), but are also frequently linked with outbreaks of foodborne illness in humans worldwide (Greig, 2010) that are typically characterized by a large number of cases and high severity of the disease (Boore et al., 2010). For this reason, microbial food safety of vegetables has
become an important priority for agri-food and public health authorities (Food and Agriculture Organization of the United Nations, World Health Organization (FAO, WHO), 2008), particularly for types of produce that are frequently eaten raw (i.e. leafy greens: lettuce, spinach, fresh leafy green herbs, etc.).

Agri-food agencies, scientists, and other stakeholders have been concurrently investing considerable efforts, both globally and locally, to map out and prioritize research in microbial food safety of vegetables using primarily expert-based opinions or research reviews (Crohn & Bianchi, 2008; European Food Safety Authority (EFSA), 2010; FAO, WHO, 2008), and various informal to semi-formal ranking tools to aid prioritization process and action plan in this area. Numerous primary research (Francis & O’Beirne, 2001; Loncarevic, Johannessen, & Rorvik, 2005; Sagoo, Little, & Mitchell, 2003) and narrative review articles have been published over the past two decades addressing various aspects of microbial contamination and control of different microbial hazards in produce throughout the farm-to-table continuum (Delaquis, Bach, & Dinu, 2007; Franz & van Bruggen, 2008; Hanning, Johnson, & Ricke, 2008; Holden, Pritchard, & Toth, 2009). Narrative reviews frequently lack rigor and reproducibility and are subject to bias due to a lack of use of reporting procedures for the identification, selection, and summary or synthesis of data from existing literature, and their overall utility to policy makers might be limited (Sargeant, Torrence, Rajic, O’Connor, & Williams, 2006; Waddell et al., 2009). Formal synthesis research methods, such as systematic review and meta-analysis, offer alternative approaches, following transparent, explicit and replicable methods to identify, to critically appraise, and to summarize (preferably quantitatively)
scientific evidence related to a very focused question (Arksey & O'Malley, 2005; Higgins & Green, 2008). These methods are used to identify, based on existing research, the most effective interventions, accuracy of diagnostic tests, consistency, and strength of risk factors, thus guiding promising intervention research and for generating evidence-based inputs (e.g. prevalence of bacteria) for complementary risk assessments and decision analysis. The resulting information is frequently used not only to guide policy development process, but also funding agencies and scientific communities. These methods, traditionally used in the human health sector, have been recently applied more extensively to the agri-food sector (Sargeant, Rajic, Read, & Ohlsson, 2006; Waddell et al., 2008; Young et al., 2009).

Scoping studies are a relatively new synthesis research format and have been extensively utilized on broader and complex policy driven topics typically spanning across a range of disciplines in medicine and allied health sectors (Davis, Drey, & Gould, 2009; Lichtenstein, Yetley, & Lau, 2008; Swanwick, Hanley, & Termansen, 2007), and recently in veterinary public health (Farrar, 2009). The main purpose of scoping study is to identify, map-out and thematically summarize the existing research, the main knowledge gaps and research needs, and in some cases to provide basic insights into the soundness (quality) of research. Thus, scoping studies are valuable synthesis research tools for gaining better understanding of complex, heterogeneous and multi-dimensional problems that require systems-based solutions (Brien, Lorenzetti, Lewis, Kennedy, & Ghali, 2010). Scoping studies follow some principles of systematic review methodology but also differ in some aspects (Table 2.1).
Our objectives were to 1) conduct a scoping study of publicly available research that investigates prevalence, risk factors, and interventions for 16 microbial hazards in leafy green vegetables, to characterize the volume, main characteristics, basic aspects of methodological soundness and reporting; and main gaps and research needs in this area; and 2) identify areas where there are currently sufficient research for future application of systematic review-meta-analysis methodology.

Methods

Team, question, protocol and definitions

The scoping study started with the establishment of a research team including members from the USA and Canada with expertise in microbiology, produce production and processing, food safety, epidemiology, and synthesis research methodology expertise. The team identified the broad question comprising of prevalence, risk factors and intervention research themes for 16 microbial hazards in leafy green vegetables. Microbial hazards were defined as bacteria, viruses, and protozoa that cause illnesses in humans associated with consumption of leafy green vegetables. An initial list of relevant microbial hazards was composed from the latest published Center for Disease Control and Prevention outbreak reports (Boore et al., 2010) and refined through the discussions with research team and two external experts (Table 2.2). Surrogates for human pathogens and food safety indicators (i.e. generic E. coli, coliforms) were included in the list. The existing definitions of WHO and USDA (FAO, WHO, 2008; Gross, Wang, & Saltveit, 32
were modified to define leafy green vegetable list (Table 2.2). Leafy green vegetables were defined as either conventional or organic, where the edible part of the plant is the leaf (usually green, but may be red or white) including those that may have been cut and packaged but otherwise remain in their original physical form and are stored, sold and consumed in a fresh (minimally processed) state. The a priori developed and pre-tested scoping study protocol included definitions (Table 2.2), procedure for literature search, study inclusion/exclusion criteria and checklists for conducting relevance screening and basic methodological assessment of relevant primary research (Table 2.3). The protocol was developed following the general principles of systematic review methodology (Sargeant, Amezcua, Rajic, & Waddell, 2005; Sargeant et al., 2006).

Search strategy

A broad list of search terms was developed by the research team in order to retrieve all citations pertaining to each of the selected microbial hazards and the leafy green vegetables. A complete list of search terms (n=62 terms) relating to population (e.g. lettuce, leafy green vegetable, etc.) and outcome (e.g. Escherichia, Norovirus, etc.) is available from the primary author upon request. Because of major changes in the leafy green industry that occurred in the last two decades, the search was limited to research published from 1990 onward. At this stage, no restrictions were imposed in terms of study design, publication language or origin of study. The search was conducted in 7 electronic databases, of broad (ISI Web of Science, BIOSIS, and PubMed) and subject-specific (CAB Direct, AGRICOLA, AGRIS, Food Science and Technology Abstracts).
nature to increase the probability of identifying potentially relevant publications. Initial (Dec 2008) and updated (Apr 2010) literature searches were performed. Citations retrieved from all databases were imported into a reference management software ("RefWorks-COS", ProQuest LLC, Ann Arbor, MI) and de-duplicated. Search verification included non-electronic searching of reference lists of four broad literature reviews (Delaquis et al., 2007; Franz & van Bruggen, 2008; Gomez-Lopez, Ragaert, Debevere, & Devlieghere, 2008; Wright, Danyluk, & Otwell, 2009) and a book chapter (Everis, 2004). Any additional, potentially relevant citations discovered through the hand-searching method which were not previously identified through electronic search, were added into the review process and processed in the same manner as electronic citations.

Abstract (RS1) and article-level relevance screening (RS2)

Through initial, abstract-based RS1 (Table 2.2), potentially relevant primary research investigating prevalence and/or risk factors and/or intervention efficacy for any of the selected 16 microbial hazards in any of the selected leafy green vegetables was identified. Non-primary research studies (e.g. narrative reviews) and studies investigating other aspects (e.g. outbreak reports or test performance studies) were intentionally excluded.

All citations deemed relevant after RS1 were procured as full articles. Through article-level RS2 (Table 2.2), the relevance of each article was confirmed. Relevant articles were assessed and categorized by the main theme (e.g. interventions intended to
eliminate or reduce microbial hazards) and by various descriptive characteristics (e.g. type of leafy green vegetable investigated or point in farm-to-table continuum), including the various types of risk factor categories (Table 2.2). Both screening levels were conducted by two independent reviewers using standardized and pre-tested checklists. Reviewer agreement (κ ≥0.8) was evaluated using 21 abstracts and 12 full articles for RS1 and RS2, respectively. Conflicts were resolved by a consensus between respective reviewers and when this was not possible, by the primary author of this study. While no language restrictions were imposed in RS1, RS2 was limited to research published in English, Portuguese, Spanish, French, Italian, Chinese, Japanese, and Slavic languages due to funding restrictions.

Assessment of methodological soundness and reporting (MSR) of relevant studies, including data extraction

In a two-phase process, each relevant study was subsequently assessed for methodological soundness and reporting (MSR) in order to characterize the overall quality of relevant primary research underpinning the topic addressed. This approach was chosen to preclude waste of resources on articles (methodological assessment and data extraction) that did not meet the minimum utility criteria. The first phase consisted of two exclusion criteria: 1) minimum sufficient reporting of data and 2) replicable laboratory protocols. Minimum sufficient data was defined as reporting of raw or unadjusted prevalence data with both the numerator and denominator reported or proportion and either numerator or denominator. For studies which employed multivariable modeling,
unadjusted effect estimates with reported sample size and measure of variability, or
adjusted effect estimates, sample size, measure of variability, and exact $P$-values were
considered essential. Studies were also excluded if the laboratory methods and materials
sections lacked sufficient detail to permit replication. This step was followed by phase
two, a basic methodological assessment and data extraction with the main aim to
characterize overall quality of relevant research underpinning the broader topic.
Additional exclusion and ranking criteria of the second phase assessment included study
specific methodological assessment and data extraction possibilities for each study (Table
2.3). These steps were conducted together by two independent reviewers using a
standardized checklist pre-tested by all participating article reviewers (n=8 articles, $\kappa$
$\geq0.8$). Disagreements were resolved by consensus or by the primary author of this study.

Study management and data analysis

Relevance screening, methodological assessment and data extraction were
conducted in two web-based software packages (SRS, TrialStat Corporation, Ottawa,
Canada; and DistillerSR, Evidence Partners Inc., Ottawa, Canada). The data were
analyzed descriptively and presented as a narrative synthesis by research themes (n=3;
prevalence, risk factor, intervention), outcome (n= 16 microbial hazards) and population
(n=17 leafy green vegetables). Only analysis of MSR data from the studies that met all
exclusion criteria was included in narrative review. Global produce related outbreak data
(received from Judy D. Greig, the Foodborne Outbreak Database, Laboratory for
Foodborne Zoonoses, Guelph, Ontario, the Public Health Agency of Canada) were
plotted against scoping data. The data for each region were not adjusted by population or gross domestic product (GDP). Descriptive analysis was performed using SAS Enterprise Guide (Cary, NC: SAS Institute Inc.) and Microsoft Excel 2007.

Results

Scoping study database

Initial and updated electronic searches yielded 7,961 citations after duplicates were removed, including 61 citations identified through hand-search verification (Figure 2.1). Out of the 6,994 abstracts excluded through RS1, 6,680 were not relevant to any of selected microbial hazards in combination with selected leafy greens, 238 were not relevant to any of three selected themes, and 76 articles were not reporting primary research or surveillance data. Among 967 abstracts considered potentially relevant through RS1, five were obtained through only hand search verification. For these, none of the keywords listed were contained in our search criteria. RS2 resulted in exclusion of 311 additional citations. Exclusion reasons are listed in Figure 2.1.

Out of 657 articles that were evaluated for descriptive characteristics, 87% were published in English (n=572 articles), 6.8% in Portuguese (n=45), 2.4% in Spanish (n=16), 1.5% in Italian (n=10), 0.8% in Chinese (n=5), 0.8% in Slavic languages (n=5), 0.5% in French (n=3) and 0.5% in Japanese (n=3).

Of all relevant articles, 30.6% (n=201) appeared in only one of the seven used electronic databases and only two papers appeared in all of the seven databases used. Cumulatively, use of five electronic databases with the highest number of relevant article
hits (BIOSIS, CAB, ISI Web of Science, FSTA and PubMed) would capture 90% of all relevant articles. Non-English articles were primarily captured through AGRIS and AGRICOLA databases. Relevant articles were published in 190 different scientific journals. Articles in English were published in 133 different journals, of which top three, *Journal of Food Protection* (n=158 relevant articles), *International Journal of Food Microbiology* (n=51) and *Applied and Environmental Microbiology* (n=30), contributed with 36% of all relevant articles. Ninety-two journals published only one relevant article each. Articles in languages other than English were published in 57 different journals, of which *Higiene Alimentar* (Brazil), *Archiv Fur Lebensmittelhygiene* (Germany), and *Revista da Sociedade Brasileira de Medicina Tropical* (Brazil) published 18, 7, and 5 relevant articles, respectively.

Overall, our scoping database of relevant articles prior to quality assessment included 36.7%, 27.7%, 15.5%, 15%, 3.5% and 1.5% from the North America, Europe, South and Central America, Asia, Africa, and Australia and New Zealand, respectively. The temporal trend comparison between number of articles published from 1990-2010 in English, Portuguese, Spanish, French and other languages, and reported disease outbreaks associated with vegetables worldwide during the same period is shown in Figure 2.2.

Overall, 314, 472 and 269 primary research studies were categorized as prevalence, risk factors and intervention studies, respectively. One article often investigated more than one category and thus the numbers add up to more than the total of 657 studies. In terms of investigated plant species, lettuce was studied in over two
thirds of articles (71.16% of articles), followed by cabbage (19.3%), green leafy herbs (15.5%) and spinach (15.2%) (Table 2.4).

The most frequently investigated microbial hazard outcomes were *Escherichia coli* (n=309 studies), *Salmonella* spp. (n=199), coliforms (n=191), and *Listeria* spp. (n=182). A combination of these four leafy greens and four pathogens accounted for 78.1% of included relevant research. A single leafy green vegetable, two, three, and more different species were investigated in 488, 109, 38 and 27 articles, respectively. Because many individual studies investigated more than one theme (prevalence, risk factors, and interventions), multiple leafy greens and pathogens are recorded multiple times in Table 2.4.

With respect to the point in the farm-to-table continuum, 24.5% (n=162) of articles were conducted at a pre-harvest (farm) level of production, 40.8% (n=269) at the processing level, and 28.9% during storage after processing and 24.7% at the retail level. The subject of the remaining studies was storage prior to processing (2%; n=13) and consumer handling (2.4%; n=16).

The country of product origin investigated in the study was reported in only half (50.9%) of the articles. In the remaining articles, the origin of vegetables was assumed to be the same as the country of the research team conducting the study and reporting the results. North America (USA and Canada) exceeded all other regions in scientific growth in this field. While number of publications on these topics had been comparable between North America in Europe up to 2008, in recent years research output from North American laboratories has doubled. Studies originating from different continents differed
in scope (prevalence, risk factor, intervention). Eighty-six percent of articles were classified into one of three main study designs: challenge trials (47.4% of studies), prevalence surveys (23.1%), and controlled trials (15.6%) (Table 2.3).

Assessment of methodological soundness and reporting (MRS)

Sixty percent (n=396) of studies were excluded through the first phase of methodological soundness and reporting assessment (MSR) (Figure 2.1). Of those, 327 studies did not contain minimum sufficient data, 14 studies did not report complete laboratory protocols, and 55 studies lacked both minimum sufficient data and complete laboratory protocols. The remaining 238 studies which passed the first phase of MSR were subsequently categorized based upon their experimental design (second phase MSR). Seven additional studies were excluded due to study-design specific criteria (Table 2.5).

Limitations identified among the studies that passed first phase assessment included randomization and representativeness of the sample (Table 2.5). Overall, less than one quarter (n=152) of all relevant studies identified met all the pre-established exclusion and descriptive MSR criteria. Among 231 studies that passed all exclusion criteria, 85% (n=197) were in English, 11.7% (n=27) in Portuguese, and 2.1% (n=5) and 0.6% (n=2) in Spanish and French, respectively.

The prevalence theme was investigated in 135 studies that passed all exclusion criteria with many of these studies presenting simultaneously risk factor or intervention research. Of these, 50 studies investigated only prevalence, 61 investigated prevalence
and at least one risk factor, 9 studied prevalence and intervention, and 15 studies investigated all three themes. Efficacy of interventions was investigated in 81 studies, including 21 with only intervention aspect. Out of 151 risk factor studies, 39 had only risk factor focus. Both risk factor and efficacy intervention were investigated in 36 studies.

**Prevalence theme**

Less than one half (43%) of all relevant studies reporting prevalence and concentration data, fulfilled MSR exclusion criteria. Their distribution by region, stage of production, investigated microbial hazard and leafy green vegetable is shown in Table 2.6. Overall, the most promising prevalence data exist for *E. coli* and *Salmonella* spp., mainly for lettuce and, to a lesser extent, for spinach and fresh leafy herbs, primarily in North America, Europe, and Central and South American regions. As a new data become available, systematic review-meta-analysis (SR-MA) could be attempted on those datasets. A moderate amount of data exists for *Listeria* spp. in cabbage (North America, Europe and Asia) and for *Giardia* spp. in watercress and lettuce from Central and South America. Information on prevalence for other pathogens in leafy green vegetables is scarce.
**Risk factor theme**

From 472 studies investigating one or more risk factors, only 32% (n=151) of publications met MSR exclusion criteria; their distribution by microbial hazard, type of leafy green vegetable and stage of production is shown in Table 2.7.

Among the 34 studies investigating contaminations risks of irrigation and wash water, other uses of water (n=5) such as ice, retail sprinkles and streams were also identified, mainly measuring *E. coli* and *Salmonella* spp. in lettuce as outcomes (Table 2.7). The use of SR-MA might be precluded due to apparent heterogeneity observed across these studies. Other pathogens such as viruses (HAV n=1; *Norovirus* n=3) or *Campylobacter* spp., *Vibrio* spp. and *Yersinia* spp. (n=2, 4, 2, respectively) were less common.

In addition to manure studies (Table 2.7), soil amendments including compost (n=2), fertilizers (n=2) and non-nutrient amendments (n=2) were investigated. The majority of manure studies were conducted on lettuce measuring *E. coli* using various study designs. Collectively, there is insufficient existing evidence to analyze the overall effect of soil contamination on transmission of pathogens on leafy greens. The role of individual worker hygiene and health in contamination of leafy greens was reported infrequently worldwide (Table 2.7). Most of these studies concerned the retail and consumer levels with a wide apparent heterogeneity observed across the studies likely precluding the use of SR-MA.
One quarter (n=58) of all studies investigating persistence or survival of microbial hazards, which was defined as increase or decrease in microorganism population levels (concentration) on leaves over time were conducted on leafy green vegetables. Most (n=35) studies investigated persistence after intervention application, for example after direct use of sanitizers (n=26) or post-processing storage (n=33). Overall, promising data are available for persistence of E. coli, Salmonella spp. and Listeria spp. in lettuce, and lack of data measuring persistence of other microbial hazards, especially viruses, and Vibrio spp.

Attachment of E. coli, Salmonella spp., Listeria spp., and coliforms on leafy greens was investigated in 10, 9, 4, and 2 studies, respectively, using a variety of mechanisms at various points of farm-to-table stages of production, precluding opportunities for robust SR-MA. Pre-harvest internalization of microbial hazards into leafy greens, seed quality and effect of storage under modified atmosphere were mainly studied in E. coli -lettuce, -spinach, and -cabbage combinations, indicating considerable heterogeneity and preclusion of SR-MA.

In seven studies that passed MSR exclusion criteria, prevalence of various microbial hazards was compared between organic and conventional produce. The role of epiphytic bacterial populations in microbial safety of leafy greens, antimicrobial resistance, and role of wildlife were examined in 2, 7 and one studies, respectively.
**Intervention effectiveness**

Of 269 studies investigating efficacy of intervention, 81 passed MSR exclusion criteria. The distribution of these studies by intervention type, microbial hazard, leafy green vegetable, and stage of production is shown in Table 2.8.

Interventions to control microbial hazards in leafy green vegetables mainly focused on the processing (86.5%; n=70) level in the produce chain, while pre-harvest interventions were investigated in only eight studies. Investigated biocontrol agents were most commonly lactic acid bacteria (n=2), or epiphytes (n=1). Other interventions included high pressure treatment, various commercial products and ultrasonication. Overall, the most abundant and consistent information is available for interventions using chlorine washes. The data for chlorine dioxide and electrolyzed water are reported in relatively small number of papers that passed the quality assessment (Table 2.8). However, due to the apparent similarities of these studies, the existing data may be sufficient to answer some questions regarding the efficacy of these sanitizers against *E. coli* in lettuce. The opposite was found for the relatively large number of relevant studies investigating effectiveness of irradiation, which provided very little quality data that could be used for further analysis. It is apparent that there is a general lack of information for interventions in other stages of farm-to-fork production process, especially pre-harvest. This precludes development of evidence-informed policy guidelines at the present time and indicates the need for research examining the efficacy of intervention options at pre-harvest.
Discussion

We created a comprehensive meta-database of the publically available literature addressing microbial hazards in leafy green vegetables and evaluated the main characteristics and methodological soundness of existing evidence. Growth in research publication has occurred in the past two decades, as microbial safety of leafy green vegetables has become increasingly important due to reported outbreaks of disease in humans associated with this food source, increased consumption, large scale production, global trade and increased consumer concern. From 1990 to present, peaks in publications on microbial hazards in leafy greens are noted in 2001 and 2009. Although scientific publishing activity in biomedical disciplines worldwide has also increased over the past two decades, the growth observed here is higher than previously reported general trends (Science-Metrix Inc., 2010). This might be related to the reported number of outbreaks and availability of research funding.

The finding that most primary research was conducted in North America and Europe is consistent with previous findings (Falagas, Michalopoulos, Bliziotis, & Soteriades, 2006; Vergidis, Karavasiou, Paraschakis, Bliziotis, & Falagas, 2005). Interestingly, Brazil (Central and South America) contributed a larger number of relevant studies (n=45) than would be expected considering this region’s overall scientific contribution in biomedical fields (Falagas et al., 2006). Recent overall economic and agri-food production growth of Brazil (OECD, FAO, 2007) may have contributed to the volume of research. The least amount of research originated from less developed regions, although these regions might benefit the most from improvements in applied research.
We did not adjust the data by region’s population or gross domestic product (GDP) nor did we attempt to rank the quality of research coming from each region by the impact factors of journals; however, previous research demonstrates that when such adjustments are made, the developing regions of the world contribute very little to the worldwide research output (Falagas et al., 2006). This is of concern for several reasons. First, the amount of published research on leafy greens by region is not proportionate to the contribution of the region to the world production of these products. For example, in 2008, China produced 12 million metric tons (mt) of produce compared to other countries (US 5.1 mt, Spain 1.1 mt, Italy 0.8 mt, India 0.8 mt, and others 3.7 mt) (Lucier & Glaser, 2010); however, only 1.4% of relevant studies originated from China. Regional discrepancy is particularly important when analyzing, interpreting and extrapolating the data because of the relative contributions of different factors such as climate, host diversity and disease prevention and control to the patterns of prevalence of human pathogens and their behavior in different geographical regions may vary significantly (Dunn et al., 2010). Many major producing countries rely on scientific information and recommendations for production from regions that are vastly different, which may jeopardize the applicability of the recommendations for these major producers.

Studies coming from different geographic regions differ in their scope. In the North America and Europe, the research focus has been heavily into fate and behavior of pathogens on leafy greens, while in Africa and Central and South America the focus has been on prevalence, pre-harvest risk factors for contamination and locally feasible mitigation options such as waste water re-use, irrigation and soil amendment.
applications, which may reflect immediate need for this type of data. The small proportion of controlled trials both in developed and developing regions could be attributed to their relatively high costs and logistical constraints. The majority of articles were published in nine subscription journals that are often not freely accessible to the public, industry, and researchers from developing countries due to the limited resources. Given the large number of studies in languages other than English that passed quality assessment, these data sources might be a valuable but overlooked source of information for evidence-informed decision making as they may enhance our understanding of the global issue and the range or applicability of interventions under a greater variety of settings.

We identified areas where a sufficient amount of data of optimal quality exists to be considered for systematic review and meta-analysis. This is primarily efficacy of chlorine based sanitizers in processing of leafy green vegetables. Similarly, existing prevalence data may be sufficient to describe the extent of problems in the main leafy greens for *E. coli, Salmonella* spp. and *Listeria* spp. in North America and Europe.

For some identified areas, such as prevalence in regions outside North America and Europe, the data are available, but there is large variability between studies (in terms of population, outcome hazard, outcome measurements, and study design, risk factor and intervention type), which precludes the use and full benefits of meta-analysis.

Inadequate reporting was identified as a major limitation in the use of this body of literature. For example, we identified topics such as irrigation water, soil amendments, and irradiation where a number of studies exist. Although these studies investigated the
same outcome in a comparable manner, due to the deficiencies in reporting, the data from only a small number of studies were available for pooled analysis. Inadequate reporting of laboratory tests was identified in over 15% of studies. This proportion is high and does not appear to be decreasing, which indicates that overall reporting of laboratory tests in this field has not improved over the last decade (data not shown). A large number of studies did not report measures of variability, precluding the use of these studies in meta-analysis. In addition, from a large number of studies reporting prevalence data, only studies designed to determine prevalence met methodological assessment criteria. This is despite the fact that prevalence and concentration measures had been collected from other study designs (e.g. pre-challenge or controlled trials). Our findings do not imply that all these studies were not correctly designed and conducted and lack valuable data, but rather that important details were not reported which limits their use in further analysis by others. Also, the weaknesses in methodological conduct or reporting might be difficult to differentiate and in some cases may be the result of brevity needs due to the journal requirements. This indicates the need that journals standardize minimum study design/results information that must be reported in the articles as part of the peer-reviewed process. Recently published scoping studies and systematic reviews addressing broader (Farrar, 2009) and focused questions in veterinary public health (Sargeant et al., 2009; Young et al., 2009) reported similar observations. Future research in food safety of vegetables will benefit from improved reporting not only from the aspect of making the data suitable for pooled analysis but would also prevent potential biases in analysis of results. Sargeant et al. (2009) reported that studies adhering to complete reporting were
less likely to find positive outcomes associated with treatment effect in veterinary medicine. It is possible that the same is true for studies investigating intervention efficacy to control microbial contamination in leafy greens. We recommend that authors attempt to adhere to reporting guidelines for their specific study design to improve the utility of their evidence. There are a number of reporting guidelines available (O'Connor et al., 2010; Sargeant et al., 2009). We identified several areas with limited amount of available information. For example, pre- and post-harvest risk factors such as facility and equipment sanitation, individual hygiene and health, seed quality and wildlife have been previously identified as potential sources of microbial contamination by experts (Wilson, Parker, Kovacs, Doohan, & LeJeune, 2009), but few papers actually investigate these risk factors. In addition, equipment sanitation, workers hygiene and health, and pre-harvest interventions for irrigation water are at the base of prevention and control points of good agricultural practices (GAPs), yet there is very little research available to support these recommendations in an evidence-informed decision making and policy development framework. In order to address these knowledge gaps properly, it is necessary that all stakeholders (government, industry, researchers, funding agencies) co-ordinate their efforts to prioritize the importance of various gaps in knowledge and prevent duplication of efforts.

Based on the comprehensive electronic and manual search, we identified relevant publically available studies. Each of seven databases used in the study contributed unique relevant articles highlighting the importance of searching multiple databases when gathering information pertinent to broad microbial food safety questions. We also found
that the relevance of a study was often not clear without the full publication which indicates the need for better, more structured, and concise reporting in titles and abstracts. This was further highlighted by the 61 references missed through electronic search due to a lack of population and outcome keywords in their respective abstracts.

One limitation of this study was that we only included selected microbial hazards, which meant that the global evidence on helminths and other parasites were not identified and mapped in this study. In addition, although we designed a very thorough and comprehensive search strategy, given the identified weaknesses in the electronic search and adequate reporting it is possible that some relevant studies still have not been identified and their addition may affect the interpretation of the results.

We encountered some challenges while performing this study, mostly related to the need for considerable resources. We also found that scoping studies are useful tool for addressing broad multi-disciplinary and multi-stakeholder questions or issues. Frequently, such questions are also underpinned with a large volume of potentially relevant studies that vary in methodological soundness and usefulness as we found in this study. Through characterization and methodological assessment of research, sound existing evidence has been identified for specific areas in a transparent manner and may aid evidence-informed decision making, as well as the identification research gaps for future resource allocation and prioritization. Importantly, the evidence map can be used to effectively identify questions/study datasets suitable for rigorous systematic review-meta-analysis and to reduce waste of resources that are needed for in-depth methodological assessment and data extraction.
Conclusions

Although a relatively young research field, food safety of leafy green vegetables is very broad in scope and studied by researchers across various disciplines investigating many issues along the farm-to-table continuum. The scientific literature in this discipline has accumulated considerably over the past two decades; however, data necessary to support the development of evidence-informed recommendations and sound policies are still missing.

While we highlight promising and consistent data for a few relevant policy related questions in this area, we report that almost 80% of identified published data in this area are not strong or even not suitable for policy and decision making due to major deficiencies in the conduct and/or reporting of primary research in this area.

We identified main knowledge gaps and future research needs. The findings of this study could be used by government and industry stakeholders, researchers and funding agencies to prioritize research efforts in a timely manner and to preclude duplication. Scoping studies are promising synthesis research tools and, in conjunction with other knowledge synthesis and translation methodologies such as systematic review-meta-analysis, mental models (Wilson et al., 2009), expert panels, stakeholder round tables (Crohn & Bianchi, 2008), risk assessment, and economic analysis (Scharff, 2010) could provide valuable contribution to informed, science-based, policy and decision making in this field.
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Figure 2.1. Flow of abstracts and articles through different steps of scoping study
Figure 2.2. The number of articles published in English, Portuguese, Spanish, French and other languages, and reported disease outbreaks associated with vegetables worldwide (Greig, 2010), 1990-2009.

Table 2.1. Differences and similarities between systematic review and scoping studies
(adopted from Brien, 2010)

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<tr>
<th>Systematic Review</th>
<th>Scoping Study</th>
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<tr>
<td>● Focused research question with narrow parameters</td>
<td>● Research question(s) often broad</td>
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<tr>
<td>● Inclusion/exclusion usually defined <em>a priori</em></td>
<td>● Inclusion/exclusion can be developed <em>post hoc</em></td>
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<tr>
<td>● Quality assessment always applied</td>
<td>● Quality assessment not an initial priority</td>
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<tr>
<td>● Detailed data extraction</td>
<td>● May or may not involve data extraction</td>
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<tr>
<td>● Quantitative synthesis often performed</td>
<td>● Synthesis more qualitative, and typically not</td>
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<td>● Formally assesses the quality of studies and</td>
<td>quantitative</td>
</tr>
<tr>
<td>generates a conclusion relating to the focused</td>
<td>● Used to identify parameters and gaps in a body of</td>
</tr>
<tr>
<td>research question</td>
<td>literature</td>
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54
Table 2.2. Summary of questions/criteria and respective definitions used in relevance screening (RS1 and RS2) for identifying and confirming relevant articles and for their categorization based on specific descriptive parameters

<table>
<thead>
<tr>
<th>Scoping stage</th>
<th>Assessed Criteria</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abstract –level relevance screening 1 (RS1)</strong></td>
<td>1. Study reporting selected microbial hazard associated with selected leafy green vegetables</td>
<td>Selected microbial hazards: Campylobacter spp., Clostridium spp., Cryptosporidium, Cyclospora, Escherichia coli, Giardia, Hepatitis A, Listeria spp., Norovirus, Salmonella spp., Shigella spp., Staphylococcus spp., Vibri spp., Yersinia spp., coliform (total coliforms, faecal coliforms or unspecified) and Enterobacteriaceae group. Selected leafy green vegetables: lettuces and baby lettuces, spinach, cabbage, fresh leafy green herbs, endive, arugula, Swiss chard, watercress, radicchio, frisée, mustard greens and spring mix.</td>
</tr>
<tr>
<td></td>
<td>2. Studies investigating prevalence and/or concentration, risk factor(s) and/or efficacy of interventions</td>
<td>Prevalence: proportion of product that is contaminated at a point in time or in a defined interval. Concentration: quantity of a selected microbial hazard on leafy green vegetables. Risk factor studies: studies that investigate any factor that may increase chances of contamination or the degree of contamination on leafy green vegetables at any stage of farm-to-table continuum. Examples were provided but not limited to six broad risk factor categories (e.g. hygiene practices, behaviors and health of food handlers and consumers). Interventions: any action applied to reduce, minimize or eliminate prevalence, contamination, or colonisation with a pathogen.</td>
</tr>
<tr>
<td><strong>Categorization of articles by themes and descriptive parameters</strong></td>
<td>1. Language</td>
<td>Language: language in which the study was published.</td>
</tr>
<tr>
<td></td>
<td>2. Publication Date</td>
<td>Publication date: year in which the study was published.</td>
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<tr>
<td></td>
<td>3. Origin of vegetable/studies</td>
<td>Origin of vegetables: geographical region of vegetable origin. Includes: USA/Canada, Europe, Asia, Central and South America (includes Mexico), Africa, Australia and New Zealand</td>
</tr>
<tr>
<td></td>
<td>a. Prevalence/concentration</td>
<td></td>
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<tr>
<td></td>
<td>b. Seed quality</td>
<td>Seed quality: microbial quality of seed used for production of leafy greens.</td>
</tr>
<tr>
<td></td>
<td>c. Water quality</td>
<td>Water quality: microbial quality of irrigation water, wash water, or other watering used in any stage of leafy green vegetable production.</td>
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<tr>
<td></td>
<td>d. Soil quality/soil amendments</td>
<td>Soil quality: effect of soil contamination on microbial hazards in leafy greens.</td>
</tr>
<tr>
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<td>e. Survival (peristence) of microbial hazard on leaves</td>
<td>Survival: persistence of microbial hazard(s) on leafy green vegetables in any stage of production.</td>
</tr>
<tr>
<td></td>
<td>f. Attachment of microbial hazards on leaves</td>
<td>Attachment: mechanisms of attachment of microbial hazard(s) on leafy green vegetables including biofilms.</td>
</tr>
<tr>
<td></td>
<td>g. Hygiene and health</td>
<td>Individual hygiene and health: hygiene practices and behaviors of food handlers and consumers in any stage of leafy green vegetable production.</td>
</tr>
<tr>
<td></td>
<td>h. Sanitation</td>
<td>Facility and equipment sanitation: contamination of equipment, utensils and transportation at any stage leafy green vegetable production.</td>
</tr>
<tr>
<td></td>
<td>i. Antimicrobial resistance</td>
<td>Antimicrobial resistance: peer-reviewed publications or publicly available reports describing antimicrobial susceptibility for any of selected 16 microbial hazards in any selected leafy greens.</td>
</tr>
<tr>
<td></td>
<td>j. Other risk factors</td>
<td>Other risk factors: factors that may increase chances of contamination or the degree of contamination on leafy green vegetables at any stage of farm to fork continuum not listed above, such as modified atmospheric packaging (MAP), interaction with other non-pathogenic microorganisms, etc.</td>
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<tr>
<td></td>
<td>k. Intervention (sanitizer, biocontrol, other)</td>
<td>Intervention: any action taken to reduce prevalence, contamination, or colonization with a pathogen. For example: sanitizers are chemical agents used for removing, reducing or preventing growth of selected microbial hazard(s) on leafy green vegetables, like chlorine, ozone, citric acid, etc. Biocontrol agents are microorganisms or direct products of a microorganism used to remove, reduce or prevent growth of selected microbial hazard(s) on leafy green vegetables.</td>
</tr>
</tbody>
</table>

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Table 2.2 continued

<table>
<thead>
<tr>
<th>Scoping stage</th>
<th>Assessed Criteria</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. Point in production chain</td>
<td><strong>Pre-harvest</strong></td>
<td>Pre-harvest or primary production: operations involved in growing cycle of leafy green vegetables from seed to the crop ready for harvest, performed at farm level in greenhouse or hydroponics.</td>
</tr>
<tr>
<td></td>
<td><strong>Harvest</strong></td>
<td>Harvest: gathering crops from the field, greenhouse or hydroponics.</td>
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<tr>
<td></td>
<td><strong>Post harvest to processing</strong></td>
<td>Post harvest to processing: actions performed after the crop is harvested until it is processed. May be performed in the field or in storage facilities, includes all means of conveyance, transportation and storage of crops before processing.</td>
</tr>
<tr>
<td></td>
<td><strong>Processing</strong></td>
<td>Processing: alteration of harvested product, however minimal (washing, cutting, etc.), whether they are performed in the plant of field or other.</td>
</tr>
<tr>
<td></td>
<td><strong>Processing to retail</strong></td>
<td>Processing to retail: storage after processing prior to retail. Includes storage and transport to retail, or storage to retail for unprocessed vegetables.</td>
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<tr>
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<td><strong>Retail</strong></td>
<td>Retail: storage and display at the point of sale, leafy greens prepared in restaurants, catering or hospitality services</td>
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<tr>
<td></td>
<td><strong>Consumer</strong></td>
<td>Consumer: post-retail events, from the point of sale to ingestion.</td>
</tr>
</tbody>
</table>
Table 2.3. Methodological soundness and reporting criteria used to evaluate relevant primary research articles that passed first phase of this assessment

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Type</th>
<th>Categories</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study design</td>
<td>Descriptive</td>
<td>Prevalence survey</td>
<td>Measures only outcome (prevalence and distribution of microbial hazard in leafy greens) at a single point in time.</td>
</tr>
<tr>
<td>Longitudinal prevalence</td>
<td></td>
<td></td>
<td>Measures outcome (prevalence and distribution of microbial hazard in leafy greens) at multiple points in time on the same population.</td>
</tr>
<tr>
<td>Challenge trial</td>
<td></td>
<td></td>
<td>Planned experiment or quasi experiment (may or may not be randomized, one or more groups) where units are artificially challenged or exposed to the studied microorganism, sampled once or longitudinally after initial treatment, lab or field trial.</td>
</tr>
<tr>
<td>Controlled trial</td>
<td></td>
<td></td>
<td>Planned experiment or quasi experiment (may or may not be randomized, one or more groups) with natural disease exposure where units are sampled once or longitudinally after initial treatment.</td>
</tr>
<tr>
<td>Cross-sectional study</td>
<td></td>
<td></td>
<td>Study done at a single point in time to investigate the prevalence and distribution of the microbial hazard in leafy greens and hypothesized risk factors within the population.</td>
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<tr>
<td>Quasi-experiment</td>
<td></td>
<td></td>
<td>Before and after trials, including prevalence measures at various points (before and after one or more stages) in the farm to consumer continuum.</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td>Any other study design not listed above, including mixed designs, cohort and case control studies.</td>
</tr>
</tbody>
</table>

| Sampling of operations: plot/farm/processing plant/grocery store | Descriptive | Random | Computer or random numbers table, a priori, stratified random sample, cluster random sample. |
| | | Reported random | Author indicates random, but randomization is not explained. |
| | | Systematic | Taking n samples at interval of x. |
| | | Purposeful | Particular subgroup of interest sampled based on a priori set criteria. |
| | | Not described | Selection criteria not described in the paper (includes convenience sampling). Operation was not selected to participate in this study or this is a lab based controlled or challenge trial. |
| No applicable | | | |

| Sampling within operations: plot/farm/processing plant/grocery store | Descriptive | Random | Computer or random numbers table, a priori, stratified random sample, cluster random sample. |
| | | Reported random | Author indicates random, but randomization is not explained. |
| | | Systematic | Taking n samples at interval of x. |
| | | Purposeful | Particular subgroup of interest sampled based on a priori set criteria. |
| | | Not described | Selection criteria not described in the paper (includes convenience sampling). Operation was not selected to participate in this study or this is a lab based controlled or challenge trial. |
| No applicable | | | |

| Representativeness of the sample population to the target population | Descriptive | Yes | At least some information provided to reflect the representativeness of the sampled population and the target population. |
| | | No | No information provided to reflect the representativeness of the sampled population and the target population. This is not a field controlled or challenge trial, quasi-experiment, case-control, cohort, cross-sectional study, prevalence survey or longitudinal prevalence study. |
| | | Not applicable | |

| Randomness of assigned treatment to the experimental unit | Descriptive | Random | Computer or random numbers table, a priori. |
| | | Reported random | Author indicates random, but randomization is not explained. |
| | | Systematic | Taking n samples at interval of x. |
| | | Not described | Selection criteria not described in the paper (including statement that it is convenience). No treatment assigned. |
| | | Not applicable | |

| Completeness of treatment protocols | Descriptive | Yes | Methods are thoroughly described and allow for replication. |
| | | No | Necessary information is missing. |
| | | In reference | Methods are referenced in another paper. |
| | | Not applicable | No treatment assigned. |

Continued
Table 2.3 continued

<table>
<thead>
<tr>
<th>Criterion†</th>
<th>Type</th>
<th>Exclusion/ Descriptive</th>
<th>Categories</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of controls and type of control group used in the study</td>
<td>Exclusion</td>
<td>None</td>
<td>Controls are from a different sampling frame, historical control or no control group was used in the study.</td>
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<tr>
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<td></td>
<td>Concurrent control</td>
<td>Controls are drawn from same sampling frame and are measured in the same timeframe as treatment group.</td>
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<tr>
<td></td>
<td></td>
<td>Before and after trial</td>
<td>The study uses the same samples as its own control.</td>
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<tr>
<td></td>
<td></td>
<td>Not described</td>
<td>Controls were not described by authors in details sufficient to make a judgment.</td>
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<tr>
<td></td>
<td></td>
<td>Not applicable</td>
<td>This is not a controlled trial, challenge trial, quasi-experiment or cohort study.</td>
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</tr>
<tr>
<td>Completeness of challenge protocols</td>
<td>Exclusion</td>
<td>Yes</td>
<td>Reported challenge organism, inoculation method, concentration, frequency and mode of inoculation (example spray, dip, etc.), drying time if applicable and storage time and temperature.</td>
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<td></td>
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<td>No</td>
<td>Some details are missing or not reported.</td>
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<td></td>
<td></td>
<td>In reference</td>
<td>Protocol is referenced in another paper.</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Not applicable</td>
<td>This is not a challenge trial.</td>
<td></td>
</tr>
<tr>
<td>Completeness of statistical test protocols</td>
<td>Descriptive</td>
<td>Yes</td>
<td>The methods were reported in sufficient detail to understand the statistical approach and reasoning.</td>
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<td>No</td>
<td>Methods and adjustments are not clear or some details are missing.</td>
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<tr>
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<td>In reference</td>
<td>Methods are referenced in another paper.</td>
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<tr>
<td></td>
<td></td>
<td>No statistical analysis</td>
<td>Statistical analysis not done.</td>
<td></td>
</tr>
</tbody>
</table>

†Descriptive criteria were not used to exclude the studies from the scoping database but to evaluate their overall quality.
Table 2.4. Number of articles for each theme leafy green vegetable-pathogen combination and their contribution to overall evidence base.

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Table 2.4 Continued

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</table>

Note that totals do not add up because one article often investigates multiple leafy greens and multiple pathogens.

* Mixed and unspecified leafy green vegetable

* Radicchio, Frizee, Mustard greens and Swiss chard

* List of all citations available upon request to author

- >30% 20-30% 10-20% 5-2% 0-1%

* Colors present percent range of total number or articles (n = 657)
Table 2.5. Methodological characteristics of studies included in second phase of methodological soundness and reporting assessment (MSR) and data extraction (n=238)

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<tr>
<th>Criteriona</th>
<th>Categories</th>
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<td>Longitudinal prevalence</td>
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<td></td>
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<td></td>
<td>Cross-sectional study</td>
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<td>Quasi-experiment</td>
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<td></td>
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<td>115</td>
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<td>Representativeness of the sample population to the target population</td>
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<td>Exclusion</td>
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<td></td>
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a Descriptive were not used to exclude the studies from consideration for further analysis.

b Studies excluded due to the unfulfilled criteria
Table 2.6. The main characteristics of prevalence study data summarized by region, outcome, study design, and investigated microbial hazard, leafy greens, and point in chain

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<tr>
<th>Study design</th>
<th>USA/Canada n=86</th>
<th>Europe n=49</th>
<th>Asia n=41</th>
<th>Central and South America n=40</th>
<th>Africa n=11</th>
<th>Australia and NZ n=4</th>
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<td>7</td>
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<tr>
<td>Concentration data only</td>
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<td>8</td>
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<td>7</td>
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<tr>
<td>Both prevalence and concentration dataa</td>
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<td>10</td>
<td>24</td>
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</table>

a Total number of relevant studies coming from each region that passed MSR assessment, total n= 231

b Number of studies by the outcome measure reported.

c Includes prevalence surveys and longitudinal prevalence studies.

d, e Studies reported prevalence of microbial hazard(s) prior to challenge/intervention.

f Study designs other than above listed include cohort, quasi-experiments, and studies of mixed design.

gh Totals do not add up because one study often investigated more than one microbial hazard.

i Out of 21 studies that investigated other microbial hazards, 10 studies from Central and South America investigated Giardia spp.

j Totals do not add up because one study often investigated more than one leafy green type.

k Out of 15 studies under other, 8 studies from Central and South America investigated watercress.
Table 2.7. The main characteristics of risk factor studies summarized by study design, investigated microbial hazard, leafy green vegetable and point in the production chain

<table>
<thead>
<tr>
<th>Studies investigating risk factors</th>
<th>Irrigation water</th>
<th>Wash water</th>
<th>Manure</th>
<th>Soil quality(^a)</th>
<th>Individual hygiene and behavior</th>
<th>Facility and equipment sanitation</th>
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<tr>
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<td>12</td>
<td>7</td>
<td>8</td>
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<td>3</td>
<td>1</td>
<td>2</td>
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<td>7</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Controlled trial ()</td>
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<td>3</td>
<td>3</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Coliforms</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>LGV</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lettuce</td>
<td>20</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>7</td>
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<td>1</td>
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<td>Fresh leafy herbs</td>
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<td>2</td>
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<tr>
<td>Farm-to-table</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-harvest(^c)</td>
<td>24</td>
<td>4</td>
<td>7</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pre-process. storage</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Processing(^d)</td>
<td>6</td>
<td>12</td>
<td>1</td>
<td>2</td>
<td>2</td>
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</tr>
<tr>
<td>Storage</td>
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<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Retail</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>5</td>
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</tr>
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<td>Consumer</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Studies investigated effect of soil quality on contamination of leafy green vegetables  

\(^b\) Study designs other than above listed, include quasi-experiments and studies of mixed design.  

\(^c\) In all studies, irrigation water, manure and soil quality was investigated in pre-harvest stage of production; in some the effect was followed throughout the chain.  

\(^d\) Wash water was investigated in processing stage of production; in some the changes were followed throughout the chain.
Table 2.8. The main characteristics of studies investigating efficacy of interventions summarized by intervention type, study design, leafy green vegetable, microbial hazards and point in production chain

<table>
<thead>
<tr>
<th>Studies investigating efficacy of interventions</th>
<th>Chlorine</th>
<th>Chlorine dioxide</th>
<th>Electrolyzed water</th>
<th>Ozone</th>
<th>UV</th>
<th>Biocontrol agent</th>
<th>H2O2</th>
<th>Peroxiacetic acid</th>
<th>Organic Acids</th>
<th>Irradiation</th>
</tr>
</thead>
</table>
| Number of studies that passed all exclusion criteria (n=81)
| 41 | 10 | 8 | 7 | 5 | 3 | 4 | 9 | 17 | 5 |
| Study design
| Prevalence survey | 3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Challenge trial | 31 | 8 | 7 | 4 | 4 | 2 | 4 | 8 | 14 | 5 |
| Controlled trial | 5 | 1 | 1 | 3 | 1 | 0 | 0 | 1 | 3 | 0 |
| Cross-Sectional | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Microbial hazard
| E. coli | 24 | 9 | 7 | 3 | 3 | 1 | 3 | 5 | 7 | 4 |
| Salmonella | 16 | 3 | 4 | 1 | 1 | 1 | 3 | 2 | 5 | 2 |
| Listeria | 9 | 4 | 2 | 1 | 2 | 2 | 2 | 3 | 7 | 3 |
| Coliforms | 11 | 0 | 1 | 2 | 1 | 1 | 0 | 2 | 5 | 0 |
| Other* | 9 | 3 | 1 | 1 | 2 | 1 | 0 | 2 | 5 | 2 |
| Lettuce | 35 | 8 | 5 | 7 | 3 | 1 | 4 | 7 | 13 | 3 |
| Cabbage | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 4 | 0 |
| Fresh leafy herbs | 8 | 1 | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| Spinach | 6 | 1 | 1 | 2 | 3 | 0 | 1 | 1 | 1 | 1 |
| Other* | 4 | 1 | 4 | 0 | 3 | 1 | 0 | 0 | 4 | 0 |
| Farm-to-table
| Pre-harvest | 4 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 |
| Pre-process. storage | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Processing | 35 | 6 | 6 | 6 | 5 | 2 | 4 | 9 | 16 | 5 |
| Storage | 11 | 4 | 2 | 0 | 0 | 1 | 0 | 1 | 2 | 1 |
| Retail | 5 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Consumer | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Organic acid refers to any organic acid used to eliminate or reduce microbial hazard and includes acetic, citric, lactic, ascorbic, lavourlinic, caprilic acid, and their salts.

* Total of all intervention studies adds up to more than 81 because some studies investigated multiple interventions.

* Study designs other than above listed, include quasi-experiments and studies of mixed design.

* Microbial hazards other than above listed.

* Leafy green vegetables other than above listed.


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Abstract

Minimally processed spinach has been recently associated with outbreaks of foodborne illnesses. This study investigated the effect of commercial minimal processing of spinach on the coliform and generic *Escherichia coli* counts and prevalence of *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp. and *Listeria monocytogenes* on two types of spinach before and after minimal processing. A total of 1356 spinach samples, (baby spinach, n=574; savoy spinach, n=782) were collected daily in 2 processing plants over a period of 14 months. Raw spinach originated from 9 farms in US and 3 in Canada.

Overall, the proportion of samples positive for coliforms increased from 53% before to 79% after minimal processing (*P*<0.001). Average total coliform counts also increased significantly after processing, especially in baby spinach (1.16 ± 0.14 log CFU/g to 2.73 ± 0.08 log CFU/g following processing; *P*<0.001). Generic *E. coli* was isolated from 8.9% of the samples (mean 1.81 ± 0.14 log CFU/g) and no difference in prevalence or
CFU counts after processing ($P > 0.1$) were observed. *E. coli* O157:H7 and *Shigella* spp. were not isolated from any of the samples. *Salmonella* spp. and *L. monocytogenes* were isolated from 0.4% and 0.7% of samples, respectively. Results demonstrate that commercial minimal processing of spinach based on monitored chlorine washing and drying may not decrease microbial load on spinach leaves as expected. Further research is needed to identify the most appropriate measures to control food safety risk under commercial minimal processing of fresh vegetables.

Introduction

Consumption of minimally processed vegetables has grown in the last decade due to the healthier diet trends and their convenience. Fresh spinach consumption alone grew by 130% between 1999 and 2006 in the USA (von Eschenbach, 2007). Despite the numerous nutritional benefits of fresh vegetable (Schroder, 2007; Wu, Hu, Willett, & Giovannucci, 2006), consumer’s choice to them in daily diet is currently influenced by the awareness of increasing number of reported outbreaks of foodborne disease (Canadian Horticultural Council, 2006).

Outbreaks of foodborne illness attributed to consumption of fresh produce have increased from 1% in the 1970s to 12% in the 1990s (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). From 1990 to 2004, fresh produce was responsible for the largest number of foodborne illness cases caused by any single commodity, constituting 21% in the CSPI (Center for Science in Public Interest) database (Smith DeWaal, Barlow, Alderton, & Jacobson, 2006). A recent outbreak linked to fresh spinach caused 205
illnesses and three deaths (Cooley, Chao, & Mandrell, 2006) and lead to significant losses for fresh spinach industry (Crepet, Albert, Dervin, & Carlin, 2007). The exact source of contamination in that outbreak was never clearly identified. The identification of a precise source of contamination of fresh spinach is often difficult as contamination can occur anywhere in the farm to fork continuum. Risk management tools used currently for controlling contamination of fresh green leafy vegetables are based on prevention, and are mostly centered on Good Agricultural Practices (GAP) for producers and Good Manufacturing Practices (GMP) for processors which provide guidelines to prevent and reduce biological contamination of fresh vegetables from known sources (Aycicek, Oguz, & Karci, 2006; California Department of Food and Agriculture, 2008; California Food Emergency Response Team, 2007). While these guidelines set up a risk management framework based on current understanding of potential hazards, there is insufficient data for validation of specific targets during minimal processing of vegetables as a measure of success for these risk mitigation strategies.

Several studies have reported coliform and *E. coli* counts on leafy green vegetables at the farm level (Crepet et al., 2007; Monterey County Agricultural Commissioner's Office, 2007; Mukherjee, Speh, Dyck, & Diez-Gonzalez, 2004) and at the retail and catering level (Harris Interactive, 2007; Sagoo, Little, & Mitchell, 2003). However, information regarding the impact of processing practices is limited. Although there are reports assessing other produce (Johnston et al., 2006; Stringer, Plowman, & Peck, 2007) only one study reporting coliform count changes before and after washing of leafy greens in an on-site packaging facility is available (Phillips & Harrison, 2005).
This study investigated the effect of a commercial minimal processing practice on the bacterial load (coliforms and generic *Escherichia coli*) and prevalence of potential foodborne pathogens (*Escherichia coli* O157:H7, *Salmonella* spp, *Shigella* spp. and *Listeria monocytogenes*) in two types of fresh spinach in a processing plant setting over a period of 14 months. The effect of seasonality and product type was also determined.

**Materials and Methods**

**Samples**

Savoy (curly leaves) and baby (flat leaves) spinach (*Spinacea oleracea*) samples were collected in two processing/packing plants from January 2006 through March 2007. Spinach originated from 9 farms in the United States (4 in Texas, and 1 each in Arizona, California, Colorado, Maryland and New Jersey) and 3 in Canada (2 in Ontario, 1 Quebec). Spinach samples were collected daily before and after minimal processing (washing and packing) in paired and unpaired fashion. Upon arrival, a 500g-composite sample of raw material was collected from every incoming truck load for each farm. Each truck load represented approximately 900 crates of ~13.6 kg for savoy and 900 crates of ~9.1 kg for baby spinach. The composite sample was made of 5 100g-samples obtained from random crates within the load. After packing, spinach samples were collected from bags or tubs from every processed lot in two processing plants. Processed lot was defined as the product processed using same incoming material originating from one incoming load, on the same processing line within the same production day (sanitation period).
Five commercial baby spinach packages were randomly collected from each lot and 100 g sample units pooled together to make one composite sample. Half of each composite sample (250g) was placed in a sterile plastic bag and transported in coolers with ice packs to a reference laboratory for blind microbiological testing. Microbiological analysis of samples was performed within 24 hours of collection at a reference laboratory.

**Minimal Processing of Spinach**

At the commercial plants, spinach processing involved washing, drying, and packing. Washing was performed in a single wash system with automatic, “demand-based”, injection of sodium hypochlorite (12.5%) and citric acid (10%) and maintained under constant oxidation-reduction potential (ORP) of -750 to -800 mV and pH of 6.8-7.0 (corresponding free chlorine concentrations between 12-25 ppm). ORP and pH electrodes were integrated to audio and electronic alarm and electronic record of values was produced in ten minutes intervals. Hand-held ORP meter was used for manual confirmation and free chlorine concentration was monitored hourly using free chlorine DPD titration test (Water Energy Technologies, Burlington, ON). The washed spinach was dried in a centrifugal dryer in plant A and in continuous air dryer in plant B, and then packed in retail bags or plastic tubs.

**Microbiological Analysis**
Both types of spinach were tested for coliforms, generic *E. coli*, *E. coli* O157:H7, *Salmonella* spp. and *Shigella* spp. Samples were initially tested only for generic *E. coli* and *E. coli* O157:H7 (n=1356). Subsequently testing for *Salmonella* spp. and *Shigella* spp (n=1311) and coliforms (n=1276) was added. In addition, minimally processed baby spinach was tested for *Listeria monocytogenes* (n=409) by qualitative and quantitative methods. All analyses were performed according to The Compendium of Analytical Methods, Health Canada (Health Canada, 1998).

Enumeration of coliforms and generic *E. coli* was performed using 3M Petrifilm™ *E. coli* count plates (Warburton, 2001). Buffered Peptone Water (225ml) was added to 25g of sample and homogenized in Stomacher (Biosciences International, Rockville, MD) for 2 minutes at 230 rpm. Samples were diluted in 0.1% peptone water, inoculated on Petrifilm and read after 24 h of incubation at 35°C.

Analysis of *E. coli* O157:H7 was conducted using an ELISA-based assay (Reveal, Neogen Corporation, Lansing, MI) on 20h broth (modified triptic soy with niacin) enrichment cultures of 50 g samples. ELISA positive samples were cultured for isolation of *E. coli* 0157:H7 as previously described (Warburton & Christensen, 2006).

The detection of *Salmonella* was conducted using an immunomagnetic separation method (Shaw, Blais, & Nundy, 2001). Suspect colonies were further biochemically and serologically confirmed following a previously described protocol (Froder et al., 2007). Presumptive positive *Salmonella* isolates were subsequently serotyped by the Health Canada, Laboratory for Foodborne Zoonoses, Guelph, ON. The detection of *Shigella* was conducted following described protocol (Liao & Fett, 2001).
Listeria spp. and Listeria monocytogenes detection was performed as previously described (Pogotto, Daley, & Farber, 2002). Confirmation of suspect colonies was performed through biochemical and serological tests as previously described (Odumeru, Belvedere, & van Donkersgoed, 2004). When specimen tested positive, the enumeration of Listeria monocytogenes in the original samples (pre-enrichment) was performed (Pogotto, Daley, Farber, & Warburton, 2001).

Statistical analysis.

Microbial counts were expressed as log\textsubscript{10} of CFU per gram. Associations between plants, processed and unprocessed spinach, product type and microbial indicator counts were investigated by using a generalized linear model. Interactions that indicated significant differences were plotted in a two-way interaction plots. The differences in proportions of samples positive for generic E. coli before and after minimal processing were compared using chi-square test. The distribution of coliform counts was analyzed using Kolmogorov-Smirnov test (Sheskin, 2000) Statistical data analysis was performed in Minitab statistical software (version 15.1, 2007, Minitab Inc., USA).

Results

In total, 1356 samples (baby spinach, 574; savoy spinach, 782) were collected daily between January 2006 and March 2007. Data for January 2007 were not available. When available, paired samples (before-after processing) were collected. Minimally processed spinach made 68.7% (n=931) of all tested samples. Four hundred and twenty
five (31.7%) samples were collected upon arrival that did not undergo further processing. Forty percent of baby spinach and 95% of savoy spinach was processed in Plant A, while the remainder was processed in Plant B. Number of samples tested per organism of interest is shown in Table 3.1.

When the results for baby spinach minimally processed in plants A and B were analyzed, no differences in coliform counts ($P=0.69$) or $E. coli$ positive samples ($P=0.52$) between the two plants were observed. No comparison was made for savoy spinach as the most of it was processed in plant A.

Significant increase in coliform counts on spinach following minimal processing was observed for both types of product ($P<0.001$). The increase of coliform for baby spinach ($1.16 \pm 0.14$ log CFU/g to $2.73 \pm 0.08$ log CFU/g). Overall, 53% ($n=215$) of unprocessed spinach was coliform-positive whereas minimally processed product tested positive for coliforms 79% ($n=688$) of the time. The observed difference was significant ($P<0.001$).

After processing 43% and 20.8% of total samples had coliform counts greater than $10^3$ and $10^4$ CFU/g, respectively. The corresponding counts for unprocessed spinach were 25% and 12.4%.

Analysis of paired data showed that coliform counts decreased after processing in only 20% of cases. For the majority of cases (80%) either no difference (27%), or an increase (53%) in coliform counts were observed after processing (Figure 3.1).

Minimal processing did not have a significant effect on the counts of generic $E. coli$ ($P=0.12$). Of total tested samples, 8.9% was contaminated with $E. coli$. The
difference between samples positive on *E. coli* before (8.4%) and after (9.0%) minimal processing, was also not significant (*P*=0.64). Most of the contaminated samples yielded very low numbers (mean of positive samples was $1.81 \pm 0.8 \log_{10} \text{CFU/g}$). Only in 2.8% (38/122) and 2.4% (33/122) of positive samples *E. coli* was present in numbers greater then $10^2$ and $10^3 \text{CFU/g}$, respectively. There was no significant difference in the *E. coli* counts detected between the two types of spinach (*P*=0.65).

Out of all samples tested for *Listeria* spp., 1.2% (5/409) were positive. *Listeria seeligieri* was detected in two samples. *Listeria monocytogenes* was isolated from three samples (0.7%), one raw and two minimally processed. Positive samples originated from the same farm and had counts of *L. monocytogenes* below $2 \log_{10} \text{CFU/g}$. All positive samples had greater than $3 \log_{10} \text{CFU/g}$ coliform counts (5/5) and were negative for generic *E. coli* and all other pathogens (5/5). No further molecular typing analyses were performed on these isolates.

*Salmonella* was detected in 0.4% (5/1311) of tested samples. All *Salmonella* isolates were recovered from savoy spinach; one from raw and four from processed spinach samples. All five samples had different origin and contained high counts of coliform greater than $3 \log_{10} \text{CFU/g}$ (5/5) and were positive for generic *E. coli* (3/5). *Salmonella enterica* Serovar Kentucky and *Salmonella enterica* Serovar Copenhagen were among serovars isolates. *Shigella* spp. or *E. coli* O157:H7 were not isolated in this study.

Throughout the study, month to month variability was observed in coliform counts and prevalence of generic *E. coli* as shown in Figure 3.2 (*P*<0.001).
Discussion

In this study, minimal processing resulted in either no change or increased coliform contamination in most (>80%) lots of processed product despite rigorous control of chlorine concentrations and ORP. Reduction of microbial populations on produce depends on the type of the produce surface being washed, type of natural microflora present (Parish, 2003) and whether the contaminant is located on the surface, in the form of biofilms or internalized. Laboratory based studies have shown that pathogens, spoilage bacteria and thermotolerant coliforms do not survive longer than 30 sec at ORP values of 665 mV or higher (Suslow, 2004). As the chlorine activity in this study was maintained under constant ORP (750-800 mV) and pH (6.8-7.0), wash water was regularly tested and was negative for coliforms and E. coli, it was unlikely that the increase occurred through contaminated input water. The equipment was sanitized daily and tested for organic residues with ATP swabs, so the likelihood that the increase can be attributed solely to cross-contamination from equipment was also low. The most logical and parsimonious explanation is that coliforms and other organisms were transferred by mixing or cross-contamination from some of the more heavily-contaminated product. It is also possible that the washing step might have enhanced coliform proliferation on the leaves by increasing water content on the leaf surfaces.

Furthermore, it is a possible that the microbial populations naturally present on leaf surfaces have different susceptibility to chlorine treatment and that chlorine treatment may create the conditions for growth of certain bacterial populations that favour or limit coliform and pathogen survival. Population dynamics of specific
coli-forms was not tested in this study. According to previously published findings, the presence of some bacterial populations on leaf surfaces or environment contributed to the observed reduction or increase of pathogens (Duffy, Whiting, & Sheridan, 1999; Western Growers, 2007). Various microorganisms have been shown to interact with pathogens in different environments at the growing, processing and storage level (Gagliardi, Millner, Lester, & Ingram, 2003; Loncarevic, Johannessen, & Rorvik, 2005; Schuenzel & Harrison, 2002; Wei, Wolf, & Hammes, 2006).

Previous reports on effects of minimal processing on microbial loads on fresh vegetables are conflicting to make an objective comparative analysis. Although no studies of spinach in commercial plants are available, results of the present study are in agreement with Johnston et al. in 2005 who found a significant increase in coliform counts in cilantro (1.4 log) and parsley (1.7 log) during processing, but the details of processing methodology were not given by the authors (Kingombe, Cerqueira-Campos, Trottier, & Houle, 2006). Likewise, increase of coliforms has been observed for other produce after washing. For instance, Gagliardi et al. (2003) reported increase of coliforms on melons from 2.5-3.5 log/g CFU before to 4-5 log CFU/g after washing. In contrast, other studies demonstrated that minimal processing, specifically washing, reduces microbial loads on fresh produce (Froder et al., 2007; Parish et al., 2003; Phillips & Harrison, 2005).

Reported reduction with chlorine washes is usually 1-2 log_{10} for pathogens on various commodities (Parish et al., 2003). Discrepancies in coliform counts and changes that occur during washing may be attributed to differences in experimental conditions.
including temperature, storage, washing treatment, and type of vegetables. In addition, most of the reported results are based on experiments performed in laboratory. Although this study was based on only two processing plants the changes in coliform contamination observed in this study indicate that similar outcomes could be observed in other plants using similar processing practices. Further studies are required to investigate the limited efficacy of these processing protocols to control microbial counts on the product.

Chlorine washes are most commonly used by fresh vegetable processing industry and their primary role is to maintain the sanitary conditions of wash water and prevent cross-contamination, while the reduction of microbial role is their secondary role. Phillips et al. (2005) reported 0.62-1.11 log reduction of coliform counts after washing spring mix with 5 ppm chlorine wash. The efficacy of chlorine washes depends on activity of chlorine (in washes and contact time and the susceptibility of the contaminating microflora.

Results from this study suggest that seasonal variations exist with a higher level of contamination in summer/fall months. This is of interest, as it seems to coincide with major US outbreaks of E. coli O157 recently reported in spinach and lettuce (Smith DeWaal et al., 2006). However, spinach processed during the summer was of a different origin than during the winter season and this may be a confounding factor for seasonal variations.

The incidence of E. coli was consistent with a number of other studies reporting E. coli present in 8.9% of lettuce samples (Mukherjee, Speh, Jones, Buesing, & Diez-
Gonzalez, 2006) and 8% on leafy green vegetables (Mukherjee et al., 2004) and in 14 out of 214 spring mix samples (Phillips & Harrison, 2005). Lower and higher prevalence was also found, for instance 1.5% in United Kingdom (Sagoo, Little, & Mitchell, 2001) and in 0.8% in the US (Kingombe et al., 2006). Higher prevalence of E. coli was reported in Turkey by Aycicek et al. (2006) from lettuce, parsley and dill (29.4%) and Mukharjee et al. (2006) from conventionally grown leafy green vegetables (24%). Garcia-Villanova Ruiz et. al in 1987 found E. coli in 86% of tested fresh vegetables. These differences can be attributed to multiple factors, such as seasonality and location. Moreover, prevalence of E. coli depends upon the type of vegetables (Mukherjee et al., 2006). Although there is some inconsistency in literature what is referred to as leafy green vegetables (Kingombe et al., 2006), in general, leafy greens and lettuce carry higher microbial loads than other fresh vegetables (Mukherjee et al., 2004).

This study demonstrated that the prevalence of foodborne pathogen contamination in commercially available product is minimal, but may exist in commercially processed products and represents a risk for serious outbreaks if post-processing proliferation is not prevented with appropriate handling and storage practices. The presented data is in agreement with previous reports for Salmonella and Listeria monocytogenes (Table 3.2). Low numbers of isolated pathogens preclude any powerful modeling of coliform and E. coli counts with pathogen presence. Despite the increase in coliform counts, generic E. coli levels were in general low and occurrence of pathogens sporadic. Relevance of coliform and generic E. coli levels on food safety of fresh produce remains unclear. These organisms are used as indicators of food safety risk in water and various foods,
where they are generally associated with pathogens that originate from similar environments (e.g., intestinal pathogens) and are also able to survive in foods (Van Kessel, Karns, Gorski, McCluskey, & Perdue, 2004). Coliform counts have been recently used to indicate food safety of raw milk in legislative microbial standards (D'Aoust & Purvis, 1998). While they certainly are indicators of microbial contamination, coliforms might not be appropriate indicators in the risk assessment of food safety of minimally processed vegetables as they might not exhibit same behavior during processing.

The interpretation of presence and number of coliform on produce is unclear at this time. The presence of coliforms does not necessarily indicate fecal contamination (Gagliardi & Karns, 2002). Nevertheless, evidence of coliforms is a measure of microbial contamination of the produce from unknown sources such as environment and possibly animal and human waste.

This study has provided valuable information on effectiveness of commercial minimal processing of spinach. It is a base for further research of complex relationships between total coliforms, *E. coli* and pathogens on leaf surfaces to determine effective measures of treatments for reduction of microbial load and removal of pathogenic microorganisms in minimal processing of vegetables.
Table 3.1. Number of spinach samples analyzed for bacterial contamination

<table>
<thead>
<tr>
<th>Spinach product</th>
<th>Commercial process</th>
<th>Total (n=*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baby</td>
<td>Savoy</td>
</tr>
<tr>
<td>Coliforms</td>
<td>564</td>
<td>712</td>
</tr>
<tr>
<td>E. coli/E. coli 0157:H7</td>
<td>574</td>
<td>782</td>
</tr>
<tr>
<td>Salmonella/Shigella</td>
<td>561</td>
<td>750</td>
</tr>
<tr>
<td>Listeria spp.**</td>
<td>409</td>
<td>0</td>
</tr>
</tbody>
</table>

*Total number of samples tested per organisms of interest

**Only processed baby spinach was tested for Listeria.
Table 3.2. Prevalence of *Salmonella* and *L. monocytogenes* in various salad vegetables.

This table shows previously reported data for *Salmonella* and *L. monocytogenes*.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Product</th>
<th>Prevalence</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>RTE vegetables</td>
<td>0.2%</td>
<td>UK</td>
<td>(Sagoo et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>0.3%</td>
<td>USA</td>
<td>(USDA, 2007)</td>
</tr>
<tr>
<td></td>
<td>Leafy greens</td>
<td>0.7%</td>
<td>USA</td>
<td>(USDA, 2004)</td>
</tr>
<tr>
<td></td>
<td>Salad vegetable</td>
<td>3.0%</td>
<td>Brazil</td>
<td>(Froder et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Salad vegetables</td>
<td>7.5%</td>
<td>Spain</td>
<td>(Ruiz et al, 1987)</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>0.4%</td>
<td>USA/Canada</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Salad vegetables</td>
<td>0.6%</td>
<td>Brazil</td>
<td>(Froder et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>1.1%</td>
<td>Norway</td>
<td>(Loncarevis et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Fresh vegetables</td>
<td>1.4%</td>
<td>France</td>
<td>(Crepet et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>2.5%</td>
<td>Australia</td>
<td>(Szabo et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>RTE vegetables</td>
<td>6.1%</td>
<td>Canada</td>
<td>(Odumeru et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>0.7%</td>
<td>USA/Canada</td>
<td>This study</td>
</tr>
</tbody>
</table>
Figure 3.1. Frequency distribution of effects of minimal processing on coliform CFU/g in paired spinach samples (n=389). Zero log₁₀ represents no change after minimal processing; negative values represent reduced CFU/g after processing; positive values represent increased coliform CFU/g after processing. The cumulative percentage of samples with increased coliform counts after processing was higher in majority of cases (P<0.01).
Figure 3.2. Monthly prevalence of *E. coli* in spinach and median coliform counts on baby and savoy spinach, 2006-2007. Data for January 2007 is not available. Note marked variation throughout the study (P>0.001), the highest levels of contamination in summer months (July-Sep) and the lowest values in winter in both years.
References


U.S. Food and Drug Administration, FDA, statement before the senate agriculture, rural development, and related agencies appropriations subcommittee. (2007).


Chapter 4- Changes in Microbial Populations on Fresh-cut Lettuce During Minimal Processing

Abstract

Foodborne illnesses linked to fresh vegetables remain an important public health concern. Minimal processing may contribute to contamination of leafy vegetables. The objective of this study was to determine the contribution of each commercial processing step to bacteriological concentration on lettuce and the potential for cross-contamination between product and food contact surfaces. In addition, microbial community profiles of leaf surfaces throughout processing were investigated. Lettuce samples and swabs of food contact surfaces were collected from seven processing steps in a commercial plant at multiple times during the processing shift. Samples were quantitatively assessed for total aerobic, coliform, and generic Escherichia coli counts. The bacterial community of minimally processed packed lettuce was profiled using T-RFLP analysis of 16S rDNA and phenotypic characterization of isolated colonies. Total aerobic and coliform counts on lettuce differed at various stages of processing ($P<0.001$). Outer leaf removal decreased the counts significantly ($P<0.001$). Initially, washing decreased both coliform and aerobic bacteria on lettuce ($P=0.006$, $P<0.001$, respectively), but both concentrations increased back to the pre-washed level during subsequent steps. Bacterial counts on food
contact surfaces were the lowest before processing began ($P<0.001$). Peak contamination on food contact surfaces was detected after two hours of processing. Contamination declined after 4-6 h of processing without intermediate sanitation interventions. T-RFLP analysis indicate the presence of 12 at least phyla/classes, but the bacterial population profiles on minimally processed lettuce were dominated by Gammaproteobacteria and Bacteroidetes. Removal of outer leaves was the single most effective step of minimal processing that impacted the microbial counts on lettuce.

Introduction

Over the last two decades, there have been several major outbreaks associated with contaminated minimally processed leafy greens (Center for Disease Control and Prevention (CDC), 2008; CDC, 2006; Grant et al., 2008), a number of which have been confirmed or speculated to originate from commercial processing plants (CDC, 2006; Warriner, Huber, Namvar, Fan, & Dunfield, 2009).

One of the purposes of minimal processing is to decrease the initial microbial load on vegetable surfaces of the incoming raw product (Gomez-Lopez, Ragaert, Debevere, & Devlieghere, 2008). This is important for the reduction of food safety risks as well as for the extension of shelf life. However, washing leafy green vegetables does not always decrease the contamination of these products (Illic, Odomeru, & LeJeune, 2008). In fact, procedures involved in minimal processing of leafy green vegetables (i.e. cutting, washing, packaging) may provide opportunities for bacterial growth following cross-contamination from either wash water, equipment and contact surfaces, or from
contaminated batches of product (Doyle & Erickson, 2008). While the reduction of 1-2 log CFU/g is generally accepted for the majority of vegetable washing techniques, we have previously reported that an increase in coliforms occurred following minimal processing in a commercial setting from almost 30% of samples from fresh bagged spinach (Ilic et al., 2008). It remains unclear which processing steps and interventions influence microbial contamination on minimally processed vegetables. *E. coli* O157:H7 persists on fresh cut lettuce during storage at 4° C (Doering, Harrison, Morrow, Hurst, & Kerr, 2009) and can survive sanitation steps after cross-contamination of fresh-cut lettuce (Lopez-Galvez, Gil, Truchado, Selma, & Allende, 2010).

In addition, the establishment of introduced pathogens on vegetables leaves is influenced by epiphytic bacteria (Warriner, Ibrahim, Dickinson, Wright, & Waites, 2003). The bacterial community profile of lettuce may be altered during various minimal processing steps. Thus, it may be informative to characterize the structure and diversity of organisms present on the surface of minimally processed lettuce and how their structure changes in response to minimal processing.

The objective of this study was to quantify the contribution of each processing step to generic *E. coli*, coliform, and total aerobic bacterial load on fresh-cut lettuce and food contact surfaces in a commercial setting and identify procedures associated with changes in microbial concentrations. Further, we determined plausible bacterial population structure of fresh-cut, minimally processed lettuce using T-RFLP analysis as a preliminary step for further studies.
Materials and Methods

*Processing plant and sample collection*

Romaine lettuce (*Lactuca sativa* var. Longifolia), grown in the US, and environmental samples were collected in locations along one minimal processing line in a large-scale vegetable processing plant in the US. Lettuce, grown in the US, shipped chilled to the processing facility was processed within three days of harvest using following steps: 1) outer leaf removal, 2) transfer to the conveyer belt, 3) cutting, 4) washing, 5) drying in centrifugal drums, 6) transfer to scale/packing, and 7) packing in polyethylene bags. Lettuce samples were collected after each step. In step one, outer leaves were collected. The first set of samples was taken in the morning at the beginning of the processing shift, and then at 3 h and 5 h of processing. All samples were taken in triplicate.

Environmental samples from food contact surfaces were collected by swabbing approximately 100 cm$^2$ of surface (Hydra-Sponge$^\text{TM}$ 3M, US) simultaneously with the collection of lettuce. Subsequent surface samples were collected from the same locations as lettuce leaves. Swabbed surfaces included: 1) cutting boards where outer leaves were removed, 2) the receiving conveyer belt, 3) the conveyer belt after cutting, 4) the conveyer belt after washing, 5) the centrifugal dryer, 6) the surface of the scale, and 7) the packing machine tube. The first set of samples was taken in the morning before the beginning of processing shift and after sanitation of the line.

Washing was performed through double cascade setting with chlorinated (sodium hypochlorite), recycled was water system, followed by one municipal water rinse. Wash
water samples were collected from the cascades in sterile, polystyrene 100 ml water bottles containing sodium thiosulfate to neutralize any chlorine (30mg/100ml Na$_2$S$_2$O$_3$).

*Escherichia coli, coliforms, and total aerobic bacteria*

The lettuce samples and the surface swabs were immediately refrigerated and processed within 12 hours of collection. Generic *E. coli*, total coliforms, and total aerobic bacterial counts were determined. Five grams fresh weight of lettuce leaves (2-3 leaf pieces) were taken from every collected sample and placed in 45 ml buffered peptone water, vortexed for 1 min and sonicated for 1 min (Aquasonic 75 HT, VWR Scientific, Mount Airy, MD). Surface sponge swabs were processed in a homogenizer (Masticator, IUL Instruments, Barcelona, Spain) for 120 sec at 8 beats per sec. Bacterial enumeration was performed using spiral plate method (WASP® II, Microbiology International, Frederick, MD). In this method 50 µl of samples is automatically dispensed on the surface of rotating plate producing a dilution by a factor of 1000 (Gilchrist, Donnelly, Peeler, & Campbell, 1977). Luria-Bertani agar (Acumedia, Lansing, MI) was used for determination of total aerobic bacterial counts and TBX agar (Acumedia) for enumeration of total coliforms and generic *E. coli*. Duplicate plates were incubated at 37 °C and counted blindly after 48 hours using a ProtoCOL® plate counter (Microbiology International, Frederick, MD).

Water samples were analyzed for generic *E. coli* and coliforms using a commercial enumeration kit (Colilert, Quanti-Tray 2000; Idexx Laboratories, Westbrook,
ME) according to the recommended procedures as previously described (Cooley et al., 2007).

*Terminal restriction fragment length polymorphism (T-RFLP) analysis*

Bacterial DNA was extracted from enriched rinsates of bagged fresh cut lettuce for community profiling. The Ultra-Clean Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) was used to extract bacterial DNA from 1 ml of overnight enrichment in tryptic soy broth at 25 ºC. Enriched rinsates were used because previous attempts to directly isolate bacterial DNA from sonicated and homogenized samples yielded a high concentration of chloroplast that competed for PCR reagents biasing the analysis. Pure bacterial DNA for sequencing was extracted from colonies grown on tryptic soy agar (TSA) (18 h incubation at 37 ºC), suspended in 100 µl of sterile water, and boiled at 95 ºC in water bath for 10 min. After the suspension was cooled on ice for 10 min, and cell debris was pelleted by centrifugation at 8,000 x g for 5 min; the DNA remained in the supernatant.

Amplification of the 16S rDNA was performed using labeled 8F (5’ AGA GTT TGA TCC TGG CTC AG 3’) and non-labeled 1492R (5’ ACG GCT ACC TTG TTA CGA CTT 3’) primers as described previously (Benitez et al. 2008). 8F-D4 primer (WellRED dye D4 fluorophore, Sigma Proligo, St. Louis, MO) was labeled for visualization of the terminal restriction fragments (TRF). PCR was performed in 25 µl reactions containing 2.5 µl of template DNA and the following: 5 µl 5x Mg-free buffer, 1.8 µl 25 mM MgCl₂, 2.5 µl 2 mM dNTPs (Promega, Madison, WI), 12.9 µl PCR water,
0.25 µl of each primer (100 pmol/µl, Sigma Proligo), 0.1 µl RNase One (25 mg/ml, Promega), and 0.3 µl GoTaq Flexi DNA polymerase (5 U/µl, Promega). Amplification was performed using PTC-200 Thermocycler (MJ Research Inc., Waltham, MA). The cycling protocol started with a 5 min initial denaturation step at 95 ºC, followed by 28 cycles of 60sec at 94 ºC, 45 sec at 54 ºC, 60 sec at 70 ºC, and ended with the final extension step of 8 min at 70º. The resulting amplicons were digested with MspI in 10 µl reactions: 5 µl template, 0.3 µl 10 U/µl MspI enzyme, 0.5 µl Buffer B, and 5.7 µl of sterile purified water (Promega). After incubation for 3 h at 37 ºC and heat inactivation at 65 ºC for 20 min, the digested DNA was precipitated with sodium acetate. The total restriction enzyme digest was amended with 1 µl of 3 M sodium acetate (pH 5.2) and 27.5 µl of cold (-20 ºC) 95% ethanol \{{17332 Westphal, A. 2011}\}. Briefly, after 10 min incubation at -80 ºC, the mixture was centrifuged (15min, 6,130 x g, 4 ºC) in a S5700 swinging-bucket rotor (Allegra 25R centrifuge; Beckman Coulter, Fullerton, CA) and the pellet rinsed twice with 70% ethanol cooled to -20 ºC. The rinse was removed by reversing the plates and spinning at 180 x g for 1 min. After drying in a laminar flow hood, the pellets were resuspended in 15 µl of sterile purified water and submitted to the Molecular and Cellular Imaging Center (MCIC) at the Ohio Agricultural Research and Development Center (OARDC, Wooster, OH) for fragment size separation on a CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA). At MCIC, 0.1 µl of each sample was mixed with 0.5 µl 600 bp size standard (CEQ DNA size standard kit 600) and 40 µl formamide solution prior to analysis.
Data containing incidence and peak height of individual TRF were generated for all samples. Analysis of fragment peaks was performed using the CEQ 8000 Genetic Analysis System software (Beckman Coulter) with a size standard of 600 nt in a quartic model and analysis parameters as previously described (Benitez et al., 2007). Briefly, binning was defined as ±1 nt for TRF ≤ 200 bp, and ±2 nt for TRF >200 bp. The relative abundance of each TRF was calculated as the proportion of the fluorescence, in relative light units, of a specific fragment to the total fluorescence of that sample. Terminal fragments of 400 nt size in non-enriched samples, previously sequenced and identified as chloroplast were excluded from the analysis. A web based program, Microbial Community Analysis III (MiCA 3), T-RFLP Analysis (PAT+), University of Idaho (Shyu, Soule, Bent, Foster, & Forney, 2007) was used to infer a plausible community structure based on identified T-RFLP data. This primer-enzyme tool analyzes observed TRF profile using the Ribosomal Database Project II (RDPII, release 10, update 26) containing 1,542,955 bacterial 16S rRNA gene sequences to define a community profile match that would theoretically account for the observed T-RFLP profile (Shyu et al., 2007). The list of plausible bacterial species that would theoretically produce these TRF was generated.

**Characterization of isolates**

Isolated bacterial colonies from minimally processed bagged lettuce were characterized using conventional tests for identification of strains following Bergey’s Manual of Determinative Microbiology (Garrity, Brenner, & Krieg, 2005). Colonies
grown on TSA after 24 h of incubation at 37 °C and 25 °C were selected according to their appearance. Two to four bacterial colonies were selected from each plate. Isolated bacterial cells were Gram stained to examine Gram reaction, cell morphology and size. Potassium hydroxide solubility test for Gram reaction was also performed (Powers, 1995). Oxidase reaction, oxidative/fermentative metabolism of glucose and lactose, and catalase production tests were performed. To determine predominant culturable Enterobacteriaceae of food safety relevance, API 20E identification system (Biomerieux, Marcy l'Etoile, France) was used to further characterize the isolates.

Sequencing of bacterial 16S rDNA

Twenty five isolates from lettuce rinsate enrichment grown on TSA at 37 °C for 18 hours were selected according to the colony appearance. One to two colonies were isolated from each plate. Colonies were identified by determination of 16S rRNA gene sequences. DNA was extracted from colonies grown on TSA (18 h incubation at 37 °C), resuspended in 100 µl of sterile water, and boiled at 95 °C in a water bath for 10 min. After the suspension was cooled on ice for 10 min, DNA was separated in the supernatant by centrifugation 8,000 x g for 5 min. Amplified 16S rDNA from isolated colonies was ligated into the pGEM-T Easy vector (Promega) and transformed into E. coli JM109 competent cells (Promega). Plasmids containing 16S rDNA were isolated by a Pure Yield miniprep kit (Promega) and submitted for sequencing (ABI Prism 3100xl Genetic Analyzer System; MCIC). Obtained sequences were analyzed using Chromas Lite 2.01
(Technelysium Pty Ltd., Brisbane QLD) and BLAST analysis was performed (Altschul et al., 1997).

Statistical analysis

Multivariable logistic regression analysis was used to investigate the association between bacterial counts and the predictors of interest (sampling time, processing step, lettuce/environmental sample) controlling for lettuce environment sampling pairs (Ailes et al., 2008). Statistical analysis was performed in Stata Statistical Software, Release 10 (StataCorp LP, College Station, TX), SAS Enterprise Guide 4.1 (SAS Institute Inc., Cary, NC) and Microsoft Excel 2007 was used for descriptive analysis of the fragment size data derived from T-RFLP.

Results

Counts before and after processing starts

Analysis of the coliform data before and after the beginning of the processing shift indicated that the extent of the coliform counts increased significantly on the surfaces within 30 min of the processing. The surfaces had the lowest counts before processing started, and the highest immediately after the beginning of the shift. Coliforms increased by 4.59 log units (95% CI of the mean= 3.54, 5.64; \( P<0.0001 \)) within 30 min of processing, and then decreased gradually thereafter. Within the next 2.5 h, coliforms
decreased by 1.18 log units (95% CI of the mean= 0.40, 1.95; \( P=0.003 \)), when lettuce and surfaces had comparable coliform counts (Figure 4.1).

Reduction of coliform counts during minimal commercial processing

Analysis of coliform data (lettuce and surfaces) indicated that the incoming product/first step of processing (removal of outermost leaves) contained the highest level of microbial contamination and that the initial processing steps until washing markedly reduced the total coliform concentration (Figure 4.2).

Outer leaves had significantly higher coliform counts (\( P=0.000 \)) than lettuce at any other later step of processing. Outer leaf removal step significantly reduced coliforms in lettuce for an average 1.32 log units (95% CI of the mean=0.39, 2.24, \( P=0.006 \)). Cutting step made no further difference in bacterial counts (\( P>0.05 \)), while counts obtained after washing were 1.7 log10 units lower (CFU 95% CI= 0.5, 2.9 CI 95%, \( P=0.006 \)). Coliform counts again increased by 2 log CFU units (95% CI=1.12, 2.74, \( P=0.002 \)) during drying and weighing. Counts of both coliforms and total bacteria on lettuce packed in bags were not clearly different (\( P>0.05 \)) to those on lettuce after outermost leaves removal (Figure 4.2). Bacterial counts in wash water were under the detection limit (10 CFU/ml).
Bacterial community profile of fresh cut minimally processed bagged lettuce

The bacterial community profile of fresh cut minimally processed bagged lettuce was characterized by several distinct terminal fragments (Figure 4.3). A total of 5,158 records, of which 3,087 records represented uncultured bacteria, were retrieved MiCA tool. Multiple cultured bacterial species produce TRF of a size equivalent to the each observed TRF (Tables 4.1 & 4.2). Bacterial phyla/classes dominant for each fragment size are shown in Figure 4.3.

All fragments identified by T-RFLP analysis clustered into two distinct size groups: a smaller range (67-83 bp) and a group of larger (486-494) fragments (Figure 4.1). Fragments of size 67 bp matched mostly the members of the Actinobacteria, although some Proteobacteria and Firmicutes were present (Table 4.1). The range 78-79 bp was mostly predicted to belong to Deltaproteobacteria. Firmicutes were predicted to be the predominant source of 80-81 nt fragments, mostly of the genus Bacillus. Representatives of a variety of phyla/classes were predicted to produce 82-83 nt size, however Betaproteobacteria were the most frequently expected based on database predictions.

Larger fragments were predicted to belong predominantly to the class of Gammaproteobacteria (Table 4.2). One third of plausible species that produced 486 bp fragment was predicted to belong to Bacteroidetes. According to the database predictions, 490 bp fragment matched mostly Pseudomonas sp., while the 494 bp fragment was predicted to belong to coliform bacteria including E. coli, Klebsiella sp, Serratia sp., Citrobacter sp. and others.
Out of 88 isolated bacterial colonies, over 50% (n=53) were preliminarily identified as *Pseudomonas* spp according to culture and biochemical tests. API 20E tests also identified *Pantoea* spp. (n=7), *Pseudomonas luteola* (n=6) cert, *Pseudomonas oryzihabitans* (n=2), *Rahnella aquatilis* (n=1), *Cedecea lapagei* (n=1), *Serratia liquefaciens* (n=2), *Enterobacter sakazakii* (n=1) and *Pseudomonas fluorescens* (n=4). *Hafnia alvei, Pseudomonas fluorescens* and *Bacillus cereus* were identified by sequencing. The majority of cultured isolates matched the species predicted by the larger (>486bp) fragments of T-RFLP analysis, with the exception of several *Pseudomonas* ssp. that were predicted by smaller TRFs (88bp).

**Discussion**

Various studies ranging from laboratory experiments to literature reviews have reported effects of washing using different sanitizers (Akbas & Olmez, 2007; Rodgers, Cash, Siddiq, & Ryser, 2004; Warriner, Huber, Namvar, Fan, & Dunfield, 2009; del Carmen Velazquez, Barbini, Escudero, Estrada, & Guzman, 2009; Gragg & Brashears, 2010; Keskinen, Burke, & Annous, 2009; Kim, Kim, & Song, 2009; Lopez-Galvez et al., 2010; Lopez-Galvez, Gil et al., 2010). The reported reductions of human pathogens on leafy green vegetables with sanitizers vary greatly: from 0.6 log CFU/g (Koseki, Yoshida, Kamitani, & Itoh, 2003; Lee & Baek, 2008), up to over 5 log CFU/g (Lang et al., 2004; Rodgers et al., 2004). Generally accepted reduction range for naturally contaminated vegetables is 1-2 log CFU/g (Doyle & Erickson, 2008). Our study shows that similar reduction can be achieved in a commercial setting; however, the
antimicrobial effect of washing appears to be short lasting as the counts increase back to their previous levels during subsequent operations (drying, weighing and packing). Significantly higher concentrations of microorganisms at the end stages of minimal processing have been previously reported (Ailes et al., 2008; Ilic et al., 2008; Odumeru, Boulter, Knight, Lu, & McKellar, 2003; Castillo & Rodriguez-Garcia, 2004).

The limitations of washing are acknowledged in literature and often attributed to factors such as organic load of wash water and cross-contamination from surfaces (Koseki et al., 2003; Koseki & Isobe, 2007; Lopez-Galvez, Gil et al., 2010). Controlled wash water parameters and undetectable bacterial counts in this study indicate low likelihood of cross-contamination via wash water. Similar was found in previous study for wash water used for processing commercial spinach (Ilic, Sanja 2008). Also, the changes in counts on surfaces over time during one sanitation cycle did not affect counts on lettuce leaves. Therefore, the elevated bacterial counts from the end product were not likely a result of cross-contamination from food contact surfaces. The reasons for the lower counts immediately after processing were not fully determined in this study. It is possible that in this study residual chlorine on the leaf surface reduced the bacterial counts during the period between sampling and culturing in the laboratory. However, residual chlorine during this period was not tested.

Outermost leaf layer removal accounted for all the reduction achieved by minimal processing in a well controlled processing line. Although this comes as no surprise, validation of outer leaf removal in a large scale lettuce processing plant was lacking. This
highlights the importance of multiple hurdles to achieve adequate microbial control in processing settings that lack a single step to eliminate bacterial hazards.

*E. coli* was not detected in this study. Although the increase of coliforms may not have significant food safety implications, the higher mean concentrations of coliforms were correlated with increased probability of detectible *E. coli* in different vegetable types (Ailes et al., 2008). The significance of coliform counts as indicators of fresh vegetable contamination is often disputed (Doyle & Erickson, 2006) as they are natural members of the epiphytic bacterial community, which we confirm here. In addition, our study is complementary to other vegetable phyllosphere studies that use different culture independent methods. Highly diverse microbiomes were reported for other leafy green vegetables: Lopez-Valasco et al. (2010) demonstrated the presence of 1000 different operation taxonomic units (OTU) on spinach leaves using pyrosequencing methods, 75% of which belonged to unknown species. In this study, 60% of plausible bacterial species producing identified fragments were classified as uncultured. However, since the enrichment was used in this study, it is expected that a number of plausible species identified in this study are well known and characterized bacteria. Some of them have clinical importance (i.e. *A. baumannii, S. pyogenes*) and food safety (i.e. *E. coli, B. cereus*) or product quality relevance. Further, some of these bacteria such as *Bifidobacteria* and*Bacteroidales*) are used as targets for microbial source tracking in water to shed a light on questions regarding the sources and transmission of fecal contamination (Stoeckel & Harwood, 2007). The limitation of the T-RFLP analysis performed in this study includes the extraction of the bacterial DNA from the enriched
rinsates, which would favor culturable fraction of organism that grew under conditions of incubation. The alternative approach to use non enriched samples, however, had a limitation of getting high amounts of chloroplast DNA. The enrichment step has been used in other studies of microbial community of tomato (Shi et al., 2009) and basil (Wetzel, Lee, Lee, & Binkley, 2010). Reported bacterial community profiles in these reports were also dominated by well characterized species.

Figure 4.1. The number of coliforms on food contact surfaces (boxplots) and on lettuce samples (diamonds) during the first five hours of processing. The difference in counts on product and surfaces was not significant (a). The letters b, c or d indicate significant differences on food contact surfaces (generalized linear model, P<0.05).
Figure 4.2. Reduction of coliform counts (estimated coefficients and 95% CI of the means) during consecutive fresh-cut lettuce processing steps in a commercial setting. The detection limit for plate counts was 10 CFU/g. The letters a, b or c indicate significant change in bacterial levels relative to incoming product (generalized linear model). The area between the dashed lines represents the bacterial levels in packed product.

* Incoming head lettuce represents outer leaves, is the reference point and therefore has no 95% confidence interval.
Table 4.1. Predicted bacterial species and taxonomic groups identified in lettuce using T-RFLP of 16S rDNA (data derived from short fragment sizes, <100bp).

<table>
<thead>
<tr>
<th>TRF (bp)</th>
<th>Bacterial species</th>
<th>Taxonomic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>66.49</td>
<td><em>Bifidobacterium</em> sp. (1)</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Variovorax paradoxus</em> (1)</td>
<td>Betaproteobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus thuringiensis</em> (1), <em>Lysinibacillus boronitolerans</em> (1), <em>Staphylococcus aureus</em> (1)</td>
<td>Firmicutes</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> sp. (1)</td>
<td>Gammaproteobacteria</td>
</tr>
<tr>
<td>78.11</td>
<td><em>Sorangium cellulosum</em> (19)</td>
<td>Deltaproteobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> sp. (2)</td>
<td>Gammaproteobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Gemmata obscuriglobus</em> (1)</td>
<td>Planctomycetes</td>
</tr>
<tr>
<td>79</td>
<td><em>Tistrella</em> sp. (3), <em>Tistrella mobilis</em> (1)</td>
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</tr>
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<td></td>
<td><em>Desulfotomaculum</em> alkaliphilum (1)</td>
<td>Firmicutes</td>
</tr>
<tr>
<td>80.48</td>
<td><em>Leifsonia xyli</em> (2), <em>Microbacteriaceae</em> (1), <em>Ornithinococcus hortensis</em> (1)</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Capnocytophaga sp.</em> (2), <em>Parabacteroides distasonis</em> (5)</td>
<td>Bacteriodetes</td>
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<tr>
<td></td>
<td><em>Geobacter metallireducens</em> (1)</td>
<td>Deltaproteobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Brevibacillus brevis</em> (1), <em>Clostridium sp.</em> (1)</td>
<td>Firmicutes</td>
</tr>
<tr>
<td></td>
<td><em>Acinetobacter sp.</em> (1), <em>Acinetobacter baumannii</em> (3), <em>Acinetobacter genomosp.</em> (1), <em>Pseudomonas</em> sp. (4), <em>P. aeruginosa</em> (1)</td>
<td>Gammaproteobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Leptospirillum</em> sp. (6)</td>
<td>Nireospiroa</td>
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<tr>
<td>81</td>
<td><em>Brevibacterium casei</em> (2), <em>Brevibacterium sp.</em> (2), <em>Kocuria rosea</em> (1), <em>Microbacterium</em> sp. (1)</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Capnocytophaga</em> sp. (1)</td>
<td>Bacteriodetes</td>
</tr>
<tr>
<td></td>
<td><em>Nitrosomonas</em> sp. (1)</td>
<td>Betaproteobacteria</td>
</tr>
<tr>
<td></td>
<td><em>Geitlerinema</em> sp. (1)</td>
<td>Cyanobacteria</td>
</tr>
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</table>

Continued
| 82.24 | Brevibacterium sp. (1) | Firmicutes |
|       | Brevibacterium sp. (1) | Gammaproteobacteria |
|       | Rhizobium leguminosarum (1) | Actinobacteria |
|       | Alphaproteobacteria |
|       | Aromatoleum aromaticum (1), Azoarcus sp. (6), Nitrosomonas sp. (4), N. europaea (1), N. eutropha (1), Petrobacter succinatimandens (2), Thauera sp. (1), T. aromatica (2) | Betaproteobacteria |
|       | Brevibacterium sp. (1) | Firmicutes |
|       | Agrobacterium sp. (1), Corynebacterium sp. (1) | Actinobacteria |
|       | Aquifex aeolicus (1) | Aquificae |
|       | Flavobacterium sp. (1), Flavobacterium psychrophilum (1) | Bacteroidetes |
|       | Alcaligenes sp. (1) | Betaproteobacteria |
|       | Bacillus gingdaonensis (1), Bacillus sp. (1) | Firmicutes |
|       | Azotobacter vinelandii (1), Pseudomonas sp. (1), Serratia marcescens (1) | Gammaproteobacteria |
Table 4.2. Predicted bacterial species and taxonomic groups identified in lettuce using T-RFLP of 16S rDNA (data derived from short fragment sizes, >100bp).

<table>
<thead>
<tr>
<th>TRF (bp)</th>
<th>TRF (bp) in silico</th>
<th>Bacterial species</th>
<th>Taxonomic group</th>
</tr>
</thead>
<tbody>
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<td>486</td>
<td>485</td>
<td><em>Adhaeribacter terreus</em> (1), <em>Chryseobacterium</em> sp. (5), <em>Flavobacterium</em> sp. (1), <em>Riemerella anatipestifer</em> (1) <em>Clostridium</em> sp. (1)</td>
<td>Bacteroidetes</td>
</tr>
<tr>
<td>486</td>
<td></td>
<td><em>Polaromonas</em> sp. (1) <em>Syntrophomonas cellicola</em> (1) <em>Cellvibrio mixtus</em> (1), <em>Pseudoalteromonas</em> sp. (1)</td>
<td>Betaproteobacteria</td>
</tr>
<tr>
<td>489.9</td>
<td>489</td>
<td><em>Leeuwenhoekiella blandensis</em> (1), <em>Zobellia uliginosa</em> (1) <em>Achromobacter</em> sp. (11), <em>Bordetella trematum</em> (1), <em>Delftia acidovorans</em> (1) <em>Duganella</em> sp. (1), <em>Kineto plastibacterium crithidii</em> (1), <em>Thiomonas</em> sp. (1), <em>Variovorax paradoxus</em> (9) <em>Nitriruptor</em> sp. (1)</td>
<td>Bacteroidetes</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td><em>Acidithiobacillus ferrooxidans</em> (1), <em>Glaciecola</em> sp. (1), <em>Microbulbifer</em> sp. (1), <em>Pseudoalteromonas</em> sp. (8), <em>Pseudomonas cichorii</em> (1), <em>Pseudomonas</em> sp. (8) <em>Streptomyces</em> sp. (1)</td>
<td>Gammaproteobacteria</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td></td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td></td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td></td>
<td>Betaproteobacteria</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td></td>
<td>Bacteroidetes</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td></td>
<td>Betaproteobacteria</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td></td>
<td>Cyanobacteria</td>
</tr>
<tr>
<td>490</td>
<td></td>
<td></td>
<td>Firmicutes</td>
</tr>
</tbody>
</table>
Table 4.2. Continued

| 494.2 1 | 494 | Castellaniella defragrans (2), Janthinobacterium sp. (1), Massilia sp. (1), Neisseria meningitidis (1), Polaromonas sp. (1) | Gammaproteobacteria |
| --- | --- | Acinetobacter sp. (2), Alcanivorax hongdengensis (1), Alcanivorax sp. (1), Alkalimonas collagenimarina (1), Aranicola proteolytica (1), Aranicola sp. (2), Beggiatoa sp. (1), C. freundii (2), Cobetia sp. (1), C. marina (2), Colwellia sp. (21), Cyanidium sp. (7), Enterobacter sp. (25), E. aerogenes (4), E. cloacae (4), Erwinia amylovora (2), Erwinia persicina (1), Erwinia pyrifoliae (6), Erwinia rhapontici (1), Erwinia tasmaniensis (2), Escherichia coli (4), Grimontella senegalensis (1), Halomonas sp. (12), Idiomarina sp. (2), Klebsiella sp. (14), K. oxytoca (30), K. pneumoniae (16), Kluvyera ascorbata (5), Kluvyera cryocrescens (1), Leclercia adecarboxylata (1), Marinobacter excellens (1), Marinobacter sp. (2), M. vinifirmus (1), Marinobacterium sp. (1), Marinomonas sp. (35), M. mediterranea (1), M. pontica (1), Neptuniibacter caesiensis (1), Oleispira sp. (1), Pantoea sp. (8), P. agglomerans (6), P. ananatis (10), P. dispersa (1), Pectobacterium carotovorum (1), Photobacterium sp. (1), Providencia sp. (2), P. alcalifaciens (1), P. rettgeri (1), Pseudalteromonas sp. (2), Pseudomonas sp. (1), Raoultella ornithinolytica (1), Raoultella planticola (1), Serratia sp. (3), Shewanella sp. (2), Vibrio sp. (1) | Betaproteobacteria |
| 495 | 495 | Alcaligenes faealis (5), Diaphorobacter sp. (1) | Gammaproteobacteria |
| | | Anabaena lemmermannii (1) Chroococcidopsis sp. (1), Nostoc sp. (1), N. sphaeroides (1) | Cyanobacteria |
| | | Enterobacter sp. (1), Escherichia coli (1), Gallibacterium anatis (1), Pantoeca ananatis (1), Pantoeca dispersa (1), Pseudoalteromonas sp. (1), Pseudomonas syringae (1), Serratia marcescens (1), Shewanella sp. (2), Vibrio tapetis (1), Xylella fastidiosa (1) | Betaproteobacteria |
Figure 4.3. Relative abundance of identified fragments by size.
References


cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology*, 27(2), 199-204.


Takeuchi, K., & Frank, J. F. (2001a). Direct microscopic observation of lettuce leaf decontamination with a prototype fruit and vegetable washing solution and 1% NaCl-NaHCO₃. *Journal of Food Protection*, 64(8), 1235-1239.


Chapter 5- Leaf-Size Dependent Survival of *Escherichia coli* O157:H7 and Epiphytic Bacteria on Minimally Processed Organic and Conventional Spinach

Abstract

Minimally processed vegetables have been associated with outbreaks of *Escherichia coli* O157:H7. Farm management practices may influence the counts and composition of epiphytic populations, including lactic acid bacteria (LAB), and thus impact the survival of pathogens on leafy vegetables. In this study, organic and conventionally-grown spinach were inoculated with green fluorescent-labeled *E. coli* O157:H7 originating from spinach to determine the survival pattern of this human pathogen in these two types of product. The counts of LAB, total aerobic bacteria, and inoculated *E. coli* (CFU/g) over the period of shelf life (14 days) were recorded. *E. coli* O157:H7 persisted throughout the storage period at refrigeration temperatures in both organic and conventional minimally processed baby spinach. Positive correlation between *E. coli* counts and LAB was observed (coefficient =0.65, *P*<0.001). Counts of both *E. coli* O157:H7 and LAB decreased (*P*<0.001) over 14 days, while total aerobic bacteria increased. Organic spinach had overall smaller leaves. *E. coli* O157:H7 counts were higher on smaller size leaves in both product types.
Introduction

Organic food markets have been growing fast (Hamm & Gronefeld, 2004), driven by increased consumers’ demand and improved accessibility at retail level (Dettmann & Dimitri, 2007). Fresh fruits and vegetable comprise over one third (36%) of all consumed organic food in the US. In the fresh vegetable market, the share of organic vegetables has increased from 4% in 2000 to 9.6% in 2008 (ERS data source). Pre-packed salads and spinach were the top two organic produce consumed (Dettmann & Dimitri, 2007).

A number of foodborne disease outbreaks associated with the consumption of leafy green vegetables in recent years have caused large numbers of illnesses and affected wide geographical areas. Leafy greens were responsible for little under half (44%) of all outbreaks and, from 1998 to 2008, 32% of cases were linked to fresh produce. E. coli O157:H7 outbreak linked to fresh spinach in 2006 caused 205 illnesses, resulting in 141 hospitalizations, 31 cases with kidney failure (Haemolytic Uremic Syndrome –HUS), and three deaths in the US and Canada in 2006 (Center for Disease Control and Prevention (CDC) 2006).

Impact of farm management practices on the microbial quality of leafy greens has been previously demonstrated (Fischer-Arndt, Neuhoff, Tamm, & Köpke, 2010; Franz, van Diepeningen, de Vos, & van Bruggen, 2005; Oliveira et al., 2010). Organic production systems are characterized by use of natural fertilizers and restrictions on chemical treatments (Oliveira et al., 2010), soils with greater microbial diversity, and smaller vegetable sizes (Reganold et al., 2010). The fate of introduced pathogens on vegetable leaves is influenced by interactions with epiphytic community (Warriner,
Huber, Namvar, Fan, & Dunfield, 2009). In previous studies, die-off rate of E. coli O157:H7 progressively declined in environment with reduced microbial community diversity (Liang, He, Powell, & Stoffella, 2011). A greater microbial diversity of epiphytic bacteria on organic leafy green herbs in comparison with conventional has been previously reported (Wetzel, Lee, Lee, & Binkley, 2010). However, it is not known whether the die-off rate of introduced pathogens is higher on the surface of organic than on conventionally grown leafy greens.

Studies determining microbiological quality of organic leafy green vegetables in comparison with conventional production at retail level are scarce (Illic et al., 2011). The majority of previous studies of organic leafy green vegetables have focused on prevalence of enteric pathogens and concentration of indicator microorganisms (Boraychuk et al., 2009; Loncarevic, Johannessen, & Rorvik, 2005; Valentin-Bon, Jacobson, Monday, & Feng, 2008) and only a few directly compare these results with conventional leafy greens (Loncarevic et al., 2005; Mukherjee, Speh, Dyck, & Diez-Gonzalez, 2004; Mukherjee, Speh, Jones, Buesing, & Diez-Gonzalez, 2006; Phillips & Harrison, 2005). The effect of organic growing practices on the bacterial and plant factors that dictate the ability of enteric pathogens to persist on leafy crops during the post-harvest production chain remains largely unexplored.

The objectives of this study were to compare the ability of E. coli O157:H7 to survive in retail organic bagged baby spinach in comparison with conventionally grown bagged baby spinach under refrigerated storage for the duration of shelf life (14 days).
Specifically, the effect of naturally present lactic acid bacteria and the effect of leaf size on survival of *E. coli* O157:H7 were assessed.

**Materials and Methods**

*Construction of fluorescent recombinant E. coli O157:H7*

Transfer of green fluorescent protein plasmid (pGFP) was performed as previously described (Fratamico, Deng, Strobaugh, & Palumbo, 1997). Plasmid containing GFP from a non-toxigenic strain of *E. coli* O157:H7 (ATCC 43888) was isolated using PureYield Plasmid Purification System (Promega, Madison, WI) according to manufacturer’s instructions. Electro-competent cells were prepared from the toxigenic *E. coli* O157:H7 strain 08-205 (CDC, spinach isolate 2006, PA). Cells were streaked from a glycerol frozen stock maintained at −80 °C to Luria-Bertani (LB) agar (Acumedia, Lansing, MI) and incubated at 37 °C for 24 h. Pure colony from the plate was transferred to 7 ml of LB broth (Acumedia). After incubation at 37 °C, 5 ml of overnight culture was inoculated into 200 ml of LB broth and the cells were grown at 37 °C and on a reciprocal shaker (300 rpm) until the OD<sub>600</sub> = 0.9. Cells were then cooled on ice for 30 min and centrifuged at 3840 x g for 15 min at 4 °C (JA-14 rotor, Beckman Coulter, Palo Alto, CA.). The bacterial pellet was resuspended in cold sterile water and the centrifugation step was repeated three times to wash the cells. Cells were finally resuspended in 500 µl of 10% glycerol. Aliquots of 40 µl were immediately frozen at -80 °C. Further, 40 µl of cold cells were mixed with 3 µl isolated pGFP or negative control (pUC19). The mixture was transferred to a cold 0.2 cm electroporation cuvette, placed in
a chilled safety chamber slide and pulsed at 25 μF, 2.5 V, 200 Ω (resulting time constant = 4.8 s; field strength = 12.5 KV/cm). Cells were resuspended immediately in 1 ml of brain infusion broth (BHI, Acumedia) warmed at 50 ºC, transferred to culture tubes and incubated with shaking at 37 ºC for 5 h and then spread onto LB agar containing ampicillin (32 mg/L) and incubated 37 ºC. The presence of pGFP was confirmed by fluorescence of colonies and the presence of shiga toxin genes, a characteristic absent in the pGFP donor strain, was detected by PCR using MK1 and MK2 primers (Karch & Meyer, 1989).

**Samples, Inoculation, and Storage**

Minimally processed pre-packed organic and conventional baby spinach was purchased in four retail stores in Ohio (1), New York (1), and Ontario, Canada (2). Five bags of conventional and five bags of organic baby spinach from each store were transported to the laboratory on ice and processed within 24 h. Two samples of approximately 30g were drawn from each bag according to the leaf size: small size sample consisting of leaves smaller than 5 cm, and large size samples consisting of leaves larger than 5 cm in length.

Recombinant *E. coli* O157:H7 with pGFP was prepared in to 25 ml of LB broth (Acumedia) at 37 ºC for 24 h with shaking to an approximate cell concentration of $10^8$ CFU/ml. The final concentration of inoculum was adjusted to $10^5$ CFU/ml with sterile phosphate saline buffer (PBS 1X, EMD, Baltimore, MD). Spinach leaves were immersed in 1 L inoculum for 1 min, allowed to drain, and then dried for 1 hour at room
temperature. The samples were repacked in polyethylene bags and stored at household refrigeration temperature (6 °C) for 14 days. Uninoculated controls were prepared in a similar fashion; samples were dipped in PBS without *E. coli* O157 inoculum.

*Enumeration of *E. coli* O157:H7, lactic acid bacteria (LAB) and total aerobic bacteria (TAB)*

The counts of *E. coli* O157:H7, total aerobic bacteria and LAB were determined immediately after the inoculation and following 2, 7, and 14 days of storage. Prior to enumeration, the bags containing inoculated spinach and controls were taken out of the fridge and kept for 1 hour at room temperature to simulate household use. Processing of spinach leaves was performed as follows: approximately 2 g fresh weight spinach leaves (1-2 leaves) were taken from each bag, placed in 18 ml buffered peptone water, and processed in a homogenizer (Masticator, IUL Instruments, Barcelona, Spain) for 120 sec at 8 beats per sec. Serial dilutions were grown on LB agar (Acumedia) for 24 h at 37 °C for the enumeration of TAB, and *E. coli* O157:H7 with the aid of UV illumination on MultiImage®II, FluoroChem®FC2 (Alpha Innotech, Santa Clara, CA). Likewise, dilutions were spread plate on de Man, Rogosa and Sharpe agar (MRS, Acumedia) for LAB, and incubated for 48 h at 37 °C in anaerobic conditions (N:H₂:CO₂ ratio 80:10:10; MiniMACS Anaerobic Workstation, Microbiology International, Frederick, MD).

*Disinfection protection assay*
After the last day of storage, inoculated baby spinach leaves were immersed in 95% ethanol for 30 sec with shaking to eliminate remaining *E. coli* O157:H7 from the surface. Leaves were then dried on sterile paper under the hood to remove excess ethanol and placed in a tube with 1X PBS. Attached cells were further dislodged by sonication for 1 min and 100 µl of sonicated samples were plated on LB agar for enumeration (24 h, 37 ºC). Glass beads were added to the sonicated spinach leaves and shaken for 5 min until macerated. 100 µl of macerated samples were plated on LB agar for enumeration (24 h, 37 ºC).

*Statistical analysis*

Multivariable linear regression model analysis was used to investigate the association between counts of *E. coli* O157:H7 and the predictors of interest (product type, leaf size, day of storage, location, and product lot) (Ailes et al., 2008). Statistical analysis was performed in Stata Statistical Software, Release 10 (StataCorp LP, College Station, TX) and MiniTAB Minitab statistical software (version 15.1, 2007, Minitab Inc., USA). Calculations were based on a confidence level equal or higher than 95% (p <0.05 was considered statistically significant). Microsoft Excel 2007 was used for descriptive analysis.

*Results*

*E. coli* O157:H7 persisted during the storage at refrigeration temperature over the period of shelf life in both organic and conventional minimally processed spinach. The
distribution of enumerated bacterial populations was normal for counts expressed as \( \log_{10} \) CFU/cm\(^2\) of leaf. Bacterial counts are shown in Table 5.1.

Average refrigeration temperature over 14 days of storage was between 5.7 and 8.2 °C (absolute range: 2-18.5°C). \textit{E. coli} O157:H7 counts decreased by 0.3 log units on day 7 \( (P=0.001, \text{ CI of the mean } 0.1-0.5) \) and 0.5 units day on 14 \( (P<0.001, \text{ CI of the mean } 0.3-0.8) \). The die-off rate was similar for both spinach types \( (P=0.624) \). Leaf size had a significant effect on \textit{E. coli} O157:H7 counts \( (P=0.005) \) with smaller leaves supporting higher counts of \textit{E. coli} O157:H7 on both product types. In general, organic pre-packed spinach had smaller leaves \( (P=0.018) \) (Figure 1).

Lactic acid bacteria levels were similar in organic and conventional minimally processed spinach but were affected by the leaf size \( (P=0.028) \). Smaller spinach leaves supported higher counts of LAB. The counts decreased during storage by 0.5 log units on day 7 \( (P<0.001) \) and by 0.4 units on day 14 \( (P<0.001) \). The decrease in the counts was observed for both uninoculated and inoculated samples. Positive correlation between \textit{E. coli} and LAB was observed (Figure 5.2).

The counts of aerobic bacteria on organic spinach leaves were overall higher than on conventional by 0.2 log units \( (P=0.006, \text{ CI of the mean } 0.06-0.4) \) throughout the study. In both products, total aerobic bacteria increased over the period of storage by 1.7 log units \( (P<0.001, \text{ CI of the mean } 1.4, 1.9) \) and 2.3 log units \( (P<0.001, \text{ CI of the mean } 2.1, 2.6) \) on day 7 and 14, respectively.

After 14 days of storage, 73 out of 96 inoculated samples tested positive for inoculated \textit{E. coli} O157:H7. Of those, 20.8% \( (n=15) \) remained positive after ethanol
treatment and sonication. After maceration, *E. coli* O157:H7 was detected in additional 11 samples (19%) of samples. The proportion of positive samples was not associated with the organic/conventional product type (chi square, *P*=0.358), nor with the leaf size (chi square, *P*=0.334).

**Discussion**

The survival of *E. coli* O157:H7 was similar on washed, bagged organic and conventional baby spinach leaves over the period of shelf life. The decline of inoculated population after two weeks was less than five fold, but it was statistically significant. In general, leaf surface is considered a poor environment for bacterial growth (Jacques, Kinkel, & Morris, 1995; Leveau & Lindow, 2001) compared to the nutrient-rich animal or human natural host environment. Lately there has been a large body of evidence in support of the hypothesis that some pathogens have adapted to grow on vegetable surface (Berger et al., 2009; Shaw et al., 2008; Warriner et al., 2009). Our study supports the previous conclusions that the pathogens can persist, but cannot grow in the phyllosphere of leafy crops (Brandl & Amundson, 2008), at refrigeration temperatures. Although recommended storage temperatures for ready-to-eat refrigerated foods are 4°C an below (FDA), our intent was to assess the ability of human pathogens to survive at previously reported storage temperatures in households (Marklinder, I. M., Lindblad, M., Eriksson, L. M., Finnson, A. M., & Lindqvist, R., 2004), and therefore the average storage temperatures in this study were slightly higher than FDA recommended.
We have also separated the effect of the leaf size from that of product type (conventional or organic) on bacterial population dynamics. Microbial population sizes for organic and conventional leafy greens were previously reported per gram of leaves (Loncarevic et al., 2005; Mukherjee et al., 2004; Mukherjee et al., 2006). However, in order to make the comparisons between the ability of human pathogens to survive on organic and conventional produce, it product types may not be sufficient to compare the only the concentrations of bacteria on the products, but rather take into consideration multiple bacterial and factors that dictate the survival of pathogens on the produce surface in processing environment. In this study organic bagged baby spinach had overall smaller leaves in comparison with conventional. This could be considered a risk factor during minimal processing of baby spinach, as the ability of introduced human pathogen, *E. coli* O157:H7 to survive on the surface of bagged minimally processed spinach was associated with the leaf size in this study. During the shelf life of the product, smaller leaves supported higher counts of *E. coli* O157:H7 than bigger leaves. Bagged baby spinach is typically sold as small leaves, possibly harvested at the young leaf age. In previous studies, the bacterial growth achieved on the young leaves pre-harvest was consistently higher than that on the older leaves, with a sevenfold difference in inoculated *E. coli* O157:H7 (Brandl & Amundson, 2008). However, smaller leaf size of organic spinach may be due to different plant nutrition regime. Smaller size of produce from organic farms than that from conventional was previously reported for strawberry fruits and leaves (Reganold et al., 2010).
Bagged spinach products go through extensive handling that may result in possible wounding and release of nutrients. The uneven distribution of nutrients and water on leaves in the package of baby spinach may provide sites that are hospitable to enteric pathogens such as *E. coli* O157:H7.

A limitation of this study, similar to the majority of studies addressing *E. coli* O157:H7 in leafy greens is the use of unrealistically high inoculum, while populations of *E. coli* O157:H7, if present in the environment, are likely very low (Mootian at al. 2009).

While increase in total aerobic bacterial counts were expected and likely reflection of the increased number of spoilage organisms, the positive correlation of LAB and inoculated *E. coli* O157:H7 counts seem to contradict the expected outcome. Lactic acid bacteria from fruits and vegetables are often investigated as biocontrol agents against plant and human pathogens (Harp & Gilliland, 2003; Scolari & Vescovo, 2004; Trias, Badosa, Montesinos, & Baneras, 2008; Trias, Baneras, Badosa, & Montesinos, 2008). However, antagonistic effects previously reported for a single strain of lactic acid bacteria (i.e. *Lactobacillus casei*, Scolari et. al. 2004; *Lactococcus lactis* or *Leuconostoc mesenteroides*, Trias et al. 2008) may not be evident when total population size is measured.
Table 5.1. Bacterial population size on organic and conventional packed spinach over the period of shelf-life.

<table>
<thead>
<tr>
<th>Day</th>
<th>Name</th>
<th>n</th>
<th>Organic CFU/cm² ±SD</th>
<th>Organic control</th>
<th>n</th>
<th>Conventional CFU/cm² ±SD</th>
<th>Conventional control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><em>E. coli</em> O157:H7</td>
<td>44</td>
<td>3.23±0.85</td>
<td>0</td>
<td>49</td>
<td>3.04±0.52</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total aerobic bacteria</td>
<td>11</td>
<td>3.92±1.04</td>
<td>4.05±1.02</td>
<td>12</td>
<td>4.35±0.86</td>
<td>4.44±0.88</td>
</tr>
<tr>
<td></td>
<td>Lactic acid bacteria</td>
<td>3.23±0.56</td>
<td>2.75±0.36</td>
<td>3.05±0.51</td>
<td>2.63±0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> O157:H7</td>
<td>3.27±0.93</td>
<td>0</td>
<td>3.13±0.71</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Total aerobic bacteria</td>
<td>44</td>
<td>5.14±0.98</td>
<td>4.77±1.02</td>
<td>48</td>
<td>5.12±0.72</td>
<td>5.12±1.01</td>
</tr>
<tr>
<td></td>
<td>Lactic acid bacteria</td>
<td>3.02±0.78</td>
<td>3.19±0.7</td>
<td>3.08±0.65</td>
<td>1.98±0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> O157:H7</td>
<td>2.9±0.85</td>
<td>0</td>
<td>2.73±0.62</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Total aerobic bacteria</td>
<td>43</td>
<td>5.99±0.95</td>
<td>4.96±1.23</td>
<td>48</td>
<td>5.82±0.83</td>
<td>6.09±0.65</td>
</tr>
<tr>
<td></td>
<td>Lactic acid bacteria</td>
<td>2.79±0.7</td>
<td>1.99±1.07</td>
<td>2.63±0.63</td>
<td>1.53±0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> O157:H7</td>
<td>2.56±0.9</td>
<td>0</td>
<td>2.46±0.74</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Total aerobic bacteria</td>
<td>47</td>
<td>6.57±0.97</td>
<td>5.99±1.03</td>
<td>48</td>
<td>6.6±0.6</td>
<td>6.28±0.77</td>
</tr>
<tr>
<td></td>
<td>Lactic acid bacteria</td>
<td>2.68±0.79</td>
<td>2.8±0.99</td>
<td>2.55±0.75</td>
<td>2.52±0.66</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1. Differences in leaf size between conventionally and organically grown pre-packed baby spinach. Letter a, b, a’, and b’ represent statistical differences within each group of leaf size.
Correlation between count of inoculated \textit{E. coli} O157:H7 and LAB on pre-packed spinach.

\textbf{Figure 5.2.} Correlation between \textit{E. coli} O157:H7 counts and LAB in organic and conventional pre-packed baby spinach during storage at refrigeration temperatures.
References


Chapter 6- Applying the Multiple-Barrier Approach for Microbial Risk Reduction in the Post-Harvest Sector of Wastewater-Irrigated Vegetables

[As published in Chapter 12, Wastewater Irrigation and Health: Assessing and Mitigating Risk in Low-income Countries, 2010]

Abstract

Post-harvest interventions are an important component of a multiple-barrier approach for health-risk reduction of wastewater-irrigated crops as recommended by the 2006 edition of the WHO Guidelines for safe wastewater irrigation. This approach draws on principles of other risk-management approaches, in particular the hazard analysis and critical control point (HACCP) concept. Post-harvest measures are of particular importance as they can address possible on-farm precontamination, and also contamination that may occur after the crops leave the farm. Key factors influencing microbial contamination along the farm to fork pathway are basic hygiene and temperature management. Both factors are, however, hardly under control in most developing countries where microbial contamination and proliferation are supported by low education, limited risk awareness, rudimentary technical infrastructure and unenforced regulations. In the face of these challenges, the most successful strategies to enhance food safety will involve interventions at multiple control points along the
production chain, with emphasis on local safety targets and innovative educational programmes fitting local knowledge, culture and risk perceptions. The WHO (2006) recommended health-based targets for risk reduction in wastewater irrigation provide the required flexibility for risk mitigation in line with the concept of food safety objectives (FSO).

Introduction

Microbial infections of foodborne origin are a major public-health problem internationally and a significant cause of death in developing countries (WHO, 1996, 2006). Underlying problems of food safety differ considerably between developing countries and the more developed part of the world (Nicolas et al., 2007). Food safety in developing countries is influenced by a number of factors. In the context of wastewater irrigation, the main concern is the increasing environmental pollution in urban areas, which does not support the changing behavior of urban consumers towards more international diets, in particular fruits and salads that are eaten raw. There is a high risk of contamination (not only affecting fruits and vegetables) at all stages of production, processing and distribution which is very difficult to control through regulations given the common constraints in supporting infrastructure (cold chain) and institutional capacities.

Approaches to address this challenge have been discussed over many years in different divisions of the WHO and FAO dealing with food quality and health. The WHO Guidelines (2006) for safe wastewater irrigation present only one of several concepts.
However, although different terminologies are used, there is considerable agreement on the best way forward.

The best known initiative is the *Codex Alimentarius* which calls upon countries to work towards international food safety and quality standards. Related recommendations, also for vegetables eaten raw, are outlined in international codes of best practices (CAC, 2003a, 2003b). Acknowledging the complexity of the current food safety situation within and across many countries, the WHO and FAO advocate targeted interventions using microbiological risk analysis as the basis for building food safety control programmes. Partly through the activities of *Codex Alimentarius* and expert consultations, both organizations have developed a series of guidelines and reports that detail the various steps in risk analysis and management (FAO/WHO, 2008; Gorris, 2005).

Quantitative microbial risk assessment can help in identifying critical control points (Seidu et al., 2008). However, in many countries the results are predictable given the general substandard situation. The critical control point concept is similar to the multiple-barrier approach recommended by different national and international agencies for drinking-water safety and also by WHO (2006) in view of wastewater-related food safety issues. The approach recognizes that while each individual barrier may be not be able to completely remove or prevent contamination, and therefore protect public health, implemented together, the barriers work to provide greater assurance that the water or food will be safe at the point of consumption.

Where a quantitative risk assessment is not available, it is still possible to set local food safety objectives (FSO) (Figure 6.1) which relate operational food safety
management to public-health goals (FAO/WHO, 2002). Health-based FSO relate to the time/point of consumption, which gives flexibility to the individual contribution of different control points to the overall risk reduction target. This flexibility also acknowledges that food chains can be very different, but nevertheless should comply with a common health-based target (Gorris, 2005). In the context of health-based targets, the ultimate goal is to have a measurable impact on specific health outcomes, such as diarrhoeal diseases. Whereas metrics and threshold targets for ‘upstream’ parameters (irrigation water quality, for example) may vary from system to system, and are often unattainable, the FSO approach is viewed as a success as long as the end result of improved health is achieved through one or more control points (barriers) before the food gets served.

The 2006 edition of the WHO Guidelines for safe wastewater irrigation (WHO, 2006) mirrors this philosophy and recommends a ‘multiple-barrier’ approach for health-risk reduction, especially where conventional wastewater treatment is not effective (Figure 6.2). Health-based targets are expressed in averted Disability-adjusted life years (DALY) (see Chapter 2). The Guidelines draw on the hazard analysis and critical control point (HACCP) system and its prerequisites: good agricultural practices (GAP), good manufacturing practices (GMP) and good hygiene practices (GHP) which are recognized by the Codex Alimentarius Commission as a costeffective way to enhance food safety at all stages of the food supply chain (WHO, 1996).

Although microbiologically polluted irrigation water is a major contributor to on-farm contamination of vegetable crops, it is only one of many risk factors in the farm to
fork continuum. There are other pathogen sources and non-pathogenic threats. Looking only at pathogens, they can contaminate the edible tissues of plants at any stage from production to consumption via soilborne, seedborne, airborne or waterborne routes. Considering the differences in existing food-production chains, with an enormous variety in structures, logistics and stakeholders, and that they will undoubtedly change rapidly, scale-up and diversify continuously, food safety management at any scale (regional, national, local, factory) is a challenge (Gorris, 2005). This shows the crucial need for multiple precautions at various pathogen barriers or critical control points. In Chapter 10, the authors introduced farm-based measures, while this chapter focuses on barriers in the post-harvest sector (Keraita, B. 2010). These address two important objectives: minimizing any existing contamination during primary production (i.e. on farm); and avoiding any additional contamination that may occur through cross-contamination and suboptimal hygiene practices during harvesting, transport, processing, marketing/handling and food preparation.

Biophysical factors affecting risk reduction

Contaminants originating from wastewater may attach to the plant surface, may be taken up by roots or may be internalized into the plant tissue elsewhere. From a food safety standpoint, the latter route is debatably less significant given the low concentrations of pathogens which can enter the tissue of healthy plants compared to what can be deposited on the surface. Although it has been shown that some human pathogens, such as Salmonella spp., can survive and grow within certain vegetables, their
replication is generally limited under these conditions (Jablonske et al., 2004; Serani et al., 2008; Shi et al., 2007; Solomon et al., 2002; Tsai and Ingham, 1997; Zhuang et al. 1995). It is more likely for pathogens to enter plants that are wounded or damaged (Aruscavage et al., 2008; Fatemi et al., 2006). The greater risk factor, in terms of quantitative pathogen exposure, is the contamination of the crop surface, especially where the surface is large, like on leafy vegetables.

Understanding the ecology of bacterial pathogens on plant surfaces can lead to the development of intervention strategies to prevent, reduce or remove contamination. Virtually any fruit or vegetable can serve as a vehicle for any pathogen, providing that the pathogen survives in high enough numbers on the product until such time as it is consumed. Common factors influencing pathogen survival include initial dose of contamination, time and environmental conditions (Table 6.1). Table 6.2 shows the die-off rate of different pathogen groups on the crop surface.

Environmental conditions play a key role in the survival of micro-organisms on plant surfaces which are subject to extreme fluctuations in temperature and moisture (Bunster et al., 1989) and related bacterial numbers and diversity (Ailes et al., 2008; Illic et al., 2008). This offers opportunities for interventions. Natural die-off of bacteria has been described as an important method to minimize safety risks by increasing the interval between the last irrigation (and contamination) and harvest to several days (Aruscavage et al., 2006; Keraita et al., 2007). Unfortunately, the same does not apply to the interval between harvest and consumption: once harvested, (leafy) vegetables begin to decay rapidly and cannot be kept on the shelf to facilitate natural die-off.
It can get even worse. As crops are transported from the farm to the table, contamination, recontamination and cross-contamination issues are gaining in importance. Consequently, instead of naturally decreasing contamination levels after harvest, several studies have shown an increase in microbial load as vegetables move from the farm to the consumer (Ailes et al., 2008; Ensink et al., 2007; Ilic et al., 2008). Only when the temperature can be controlled and kept low can longer intervals allow for bacterial die-off; but where temperature cannot be controlled, extended time between harvest and consumption may support an increase in bacterial population numbers rather than a decrease (Figure 6. 3).

Options for risk reduction along the contamination pathway

Harvest

Different paths of contamination are possible during the harvest of leafy green vegetables (Franz and van Bruggen, 2008; Hope et al., 2008; McEvoy et al., 2009). While in more developed countries most concerns are addressed through standardized protocols and mechanized field operations, in developing countries basic hygiene is often violated due to the high dependency on manual labor combined with the lack of clean water or other resources and/or education.

Harvest is a key step along the contamination pathway as it involves the injury of plant tissues. As discussed above, cut surfaces are ideal sites for pathogen attachment and may also serve as an entryway of pathogens into the deeper tissues of the plant where they cannot be disinfected or washed away (Aruscavage et al., 2008). The cleaning and
sanitization of equipment used during harvest is an important requirement (McEvoy et al., 2009), but of limited applicability in smallholder farms in developing countries where water for cleaning might not be available and tools are permanently in contact with hands, crops and soil. However, as mentioned above, plant injury and internalization of pathogens at harvest are only noteworthy where surface contamination is not a larger risk factor.

During harvesting and immediately after, fresh vegetables are also exposed to potential cross-contamination from the soil surface, other agricultural inputs (e.g. fresh manure) and handlers. It is important to implement basic sanitary practices to prevent contamination at this level. Using baskets or plastic sheets to avoid contact between utensils and produce and the ground or other potentially unsafe sources of contaminants can greatly contribute to the reduction of the risks of contamination during harvest.

In both northern and southern vegetable production systems, emphasis is often placed on performing parts of the processing while the crop is still on the farm. For example, in-field coring and packaging of lettuce heads in the USA has become a common industry practice (McEvoy et al., 2009). Likewise, in West Africa, it is common practice for vegetable sellers to buy their crops on the farm. This allows them to choose the best-looking ones. Still on the farm, they remove soil particles from freshly harvested vegetables (e.g. carrots, salad greens and cucumbers) by washing them in the streams or ponds usually used for irrigation, as reported, for example, in Niger, Benin, Burkina Faso and Senegal (Klutse et al., 2005). These water sources are often highly contaminated, which undermines growers’ efforts to avoid contamination and poses a significant risk to
the consumer as well as all stakeholders involved in subsequent crop handling (Hope et al., 2008). Raising awareness about microbial hazards among traders is required, as is the provision of acceptable alternative water sources in which to wash vegetables.

*Transport and storage*

The main risk factor for increased microbial loads on fresh vegetables during transportation and storage is elevated temperatures over extended periods of time. In many developing countries, there is, however, still a general lack of cool transport and storage. This explains why some crops, especially the most perishable such as lettuce, are often grown in the city close to the point of sale. This urban vicinity, on the other hand usually results in irrigation with contaminated surface water (Drechsel et al., 2008).

The lack of cool storage requires fast transport from farm to retail and exact prediction of quantities to be sold to avoid leftovers. In some countries, intermediate traders are gaining ground to supply to large outlets such as supermarkets. A common bottleneck in this situation is the heat exposure of vegetables already packed in closed plastic bags during transport and intermediate storage, i.e. before the supermarket is reached.

The rate and extent of microbial growth in fresh produce depend mainly on the initial microbial load and time/temperature exposures. In general, lower storage temperatures ensure a longer post-harvest life for fresh fruits and vegetables (Nunes, 2008). Storing produce in the shade is one of the few methods available to keep produce cooler where refrigeration in not feasible.
Another risk factor typical for developing countries is the lack of dedicated transport vehicles. Usually, market traders or farmers hire taxis or mini-vans which are used at other times for the transport of commuters, small livestock or other goods, which increases the general risk of cross-contamination.

In northeastern India, farmers often transport their produce from the field to the market by bullock/buffalo cart, as it is the cheapest available transport. While on-farm packaging practices are almost nonexistent, some farmers use straw for crops such as tomatoes as a cushioning material to reduce mechanical damage. Traders further pack the tomatoes in smaller paper cartons with no ventilation and send them to distant markets, with a large proportion of the products damaged and decayed by the time they reach the consumer, which increases food safety risks (Directorate of Research (Agri) Assam Agricultural University, 2005).

*Processing and marketing*

Handling, processing and packaging of leafy green vegetables is carried out differently in diverse environments throughout the world. International standards as supported by the *Codex Alimentarius* remain in many developing countries only a long-term target as local conditions, education, regulations and infrastructure (cooling, transport means, etc.) including monitoring cannot yet match what is possible in more developed countries. The first processing step of fresh vegetables in local African and Asian market chains is often the removal of soil particles and dust to improve their general appearance and market value. In Ghana, for example, the simple removal of
(‘badlooking’) outer vegetable leaves in markets reduced the coliform counts by 0.5 log unit (lettuce) to 1 unit (cabbage) (M. Akple, pers. communication). Cutting the cabbage into smaller units, on the other hand, increased the surface area and coliform counts, which shows that every manipulation of fresh vegetables down the processing chain may be a source of contamination if prevention measures, such as cleanliness of processing equipment and the surroundings, including hygiene, health and adequate training of the involved staff, are lacking.

As in all stages of production and processing, workers may be the key sources of produce-contamination with pathogens, primarily viruses (Norovirus, Hepatitis A) and bacteria (Shigella, Salmonella, etc.). The two main and most basic steps for risk reduction would be to provide sufficient handwashing facilities and to avoid having ill individuals harvest or handle produce. However, both recommendations face significant challenges in developing countries. On the one hand, labor associations covering the health protection of formal and informal restaurant, vendor and catering staff are usually nonexistent. On the other hand, the urbanization rate has outpaced development of sanitary infrastructure. For example, a market survey in Ghana’s capital, Accra, found that only 31 per cent of the urban markets have a drainage system, 26 per cent have toilet facilities and 34 per cent are connected to pipe-borne water (Nyanteng, 1998). These data are very similar to those reported from a global survey on street-vended food (WHO, 1996).

*Final point of sale*
Where the lack of local infrastructure constrains the provision of acceptable hygienic conditions, as described in the previous section, relocation of markets or food stalls is often discussed, especially those that are informal. However, the WHO (1996) noted correctly that street-food vendors are, in many countries, part of the social and cultural fabric of their communities and, therefore, an effort should be made to keep them as close to their current business sites as possible, even though some sanitary facilities may not be available. The reasons are at least twofold:

1. The provision of new sites away from traditional locations often results in business disadvantages, thus there is low adoption and/or an informal reappearance of the stands near the former location.

2. Although better sanitary conditions might reduce the number of risk factors, they may not automatically impact on raw material contamination, cross contamination, personnel hygiene behavior, poor food preparation practices or hot and cold holding capacity.

Consequently, relocation should not be seen as a panacea for resolving the problems of low food safety. Indeed, risk mitigation has to start on farm (see Chapter 10) and continue during harvest.

The last point of sale can be a street-market, a supermarket or a restaurant offering, for example, a fresh salad. Although the standards of these entities vary greatly in developing countries, general food safety considerations are similar and again are much dependent on the ability to keep the produce under low temperatures and well protected from contamination. Especially in hot climates, it is often impossible to
conserve unsold leafy vegetables for the next day because of product quality deterioration. Even during the day, water is often used for refreshing or rehydrating (crisping) fruits and vegetables on display. Changing this water once during the day can already decrease the average fecal coliform counts on lettuce by up to 1 log unit, as a comparative analysis has indicated (Drechsel et al., 2000). However, in many developing countries where it is not easy to change water, vegetables are refreshed and washed over the day with the same water, which can lead to severe cross-contamination (Amoah et al., 2007a).

In theory, the use of chlorine tablets could help, but if solutions used for decontamination are not regularly changed, such processing water may itself become a source of contamination. Therefore, clear instructions on dosages and frequencies of water replenishment and disinfectants should be provided and followed. More important is the need to address the motivation for washing or refreshing vegetables in retail. The most obvious motivation is to display ‘fresh’ produce, which reflects customers’ preferences and criteria for purchase; this does not automatically translate into ‘safe’ products but could be a starting point for awareness campaigns. Such campaigns should be based on local perception studies. In Kumasi, Ghana, public-health students worked as interns over several weeks in eating places of various types (street kiosks, canteens, restaurants), observing behavior and trying to understand limitation and opportunities to increase food safety (Rheinländer et al., 2008). According to their findings, consumers avoided food safety risk by assessing the neatness and trustworthiness of vendors.
Vendors were also found to emphasize these attributes while ignoring basic food safety practices.

Consumption at home and in restaurants

Diets vary and the consumption of raw salads is not common in every country or region. However, fresh leafy greens are increasingly eaten in urban centres, e.g. in sub-Saharan Africa, as a modern complement to rice-based fast food. In Ghana, for example, more than 90 per cent of the lettuce produced enters the street-food sector; in Accra alone, at least 200,000 residents of various socio-economic classes consume lettuce or cabbage every day. Most of this produce is grown in urban and peri-urban agricultural plots irrigated with polluted water (Amoah et al., 2005, 2007a; Obuobie et al., 2006).

While markets, transport and retail can be influenced (and, in some countries, also regulated) by governmental guidelines and control measures, consumer behaviour can hardly be controlled through formal regulations (Fischer et al., 2007). On the other hand, if risk awareness is provided, consumers should have a high incentive to practice safe food handling behaviors because of the direct and immediate impacts on their own health. The challenge is to understand if this awareness is actually influencing behavior and on what kind of information it is based. Surveys of 210 restaurants and 950 households in seven countries across West Africa showed, for example, that vegetable-washing is common in 56–90 per cent of the urban households and 80–100 per cent of the restaurants (Amoah et al., 2007b; Klutse et al., 2005).
The reasons for washing varied between cities and countries, broadly depending on educational and economic standards, the availability of certain disinfectants and local traditions. In some households, vegetables were washed primarily to remove dirt, sand, dust and, more seldom, chemical farming residues. In other households and restaurants it was performed explicitly to reduce the risk of pathogens and diarrheal diseases (Amoah et al., 2007b; Klutse et al., 2005). The most common disinfectants used in the restaurants throughout Francophone West Africa were bleach (Eau de Javel®) (55 per cent of cases) and potassium (K) permanganate (31 per cent), followed by salt, lemon or soap. In Anglophone Ghana, the use of bleach was unknown and the general awareness level related to pathogen contamination appeared to be much lower (Amoah et al., 2007b). Amongst the lower classes in the selected Francophone cities, there was a clear tendency for only water or water with salt, soap or lemon juice to be used, while in middle and upper class households and restaurants the use of bleach or permanganate appeared to be prevalent (Figure 6.2).

In Ghana, various salt and vinegar solutions are dominantly used besides cleaning in water only. Salt is preferred to vinegar for cost reasons, but both appeared highly ineffective in the low concentrations or contact times commonly used (Amoah et al., 2007b). Also Rosas et al. (1984) stressed that common washing practices very often do not reduce the coliform counts to safe levels. There can be large differences depending on contact time and sanitizer (Table 6.3). The observed differences in the knowledge of appropriate sanitizers between Francophone and Anglophone countries in West Africa call for an engagement of the private sector in food safety campaigns.
Washing can also remove helminth eggs especially with good agitation and rubbing of the leaves. When washing in a bowl was compared to washing under running water (independent of sanitizing solution used) the latter was more effective in egg reduction. Washing in a bowl reduced the helminth egg population by half and sometimes more, while running water reduced the contamination level from the usual eight to nine eggs to one egg per 100g lettuce wet weight (Amoah et al., 2007b).

When it comes to internalized pathogens or pesticides on vegetable surfaces even thorough washing has its limitations (Figure 6.5).

**Education of stakeholders in post-harvest risk reduction**

Education in food safety is critical for implementation of risk reduction and mitigation measures during post-harvest production of fresh produce in both developed and developing countries. In general, stakeholders at every level need to be included in food safety education, including policy-makers. In the developing world there is a special need to improve both processors’ and consumers’ understanding of food safety.

Educational campaigns should target the following three groups:

- Processors: in regions where cost is the main barrier to implementing safe practices, education efforts should aim to inform the stakeholders about available low-cost alternatives that can be successfully implemented locally. Educational programs should also include cost–benefit comparisons and take into account cultural preferences and patterns of behavior. Aside from conventional training workshops, there are also other educational approaches
which try, for example, to show the invisible risk moving along the pathogen pathway (Figure 6.6).

- Policy-makers: at the national and international level, the *Codex Alimentarius*, supported by the FAO and WHO, probably has the best potential and network to foster awareness and influence decision-making. Care has to be taken to support countries with appropriate steps towards achieving the international standards.

- Consumers: the educational activities may target the general consumer audience on various levels of society, such as schoolchildren, women, households, etc. As the main considerations differ from country to country, it is crucial to understand the barriers in each region and possible opportunities in order to implement a successful food safety educational campaign. As the West African example showed, sometimes certain easy-to-buy disinfectants might simply not be known.

However, the step from increased awareness to actual behavior change is not an easy one and might require certain triggers and incentives as described in Chapter 16.

**Conclusions**

Due to poverty-related poor sanitary conditions in most developing countries, it is difficult to maintain appropriate hygienic standards in support of food safety. The enforcement of unrealistic standards, on the other hand, would neither be effective nor address the core of the problem, which is often the lack of understanding of hazards and safe practices (Nicolas et al., 2007). Therefore, regulations based on international
standards have very limited local application potential, although they are useful long-term goals. In addition, the application of the common HACCP concept is challenged by the multitude of existing sanitary hazards likely to affect the condition of the food along the farm to fork pathway as well as the many individual entities concerned, who often lack the collective organization, education, risk awareness and resources to undertake HACCP studies. While, for example, priority-setting via qualitative microbial risk assessment (QMRA) would be desirable, the common lack of resources limits its application. What is required under these circumstances is an integrated but flexible approach, keeping in mind what is realistically possible, and the awareness and motivation level of all the concerned parties. Agreeing on local FSO and striving for continuous improvement in the levels over time are key elements of an adapted concept. This mirrors the WHO (2006) recommended health-based targets for risk reduction in wastewater irrigation, which are, like the FSO, related to the time of consumption, i.e. the end of a food chain.

Critical control points remain important to avoid and/or reduce contamination. The studies in West Africa by Amoah et al. (2007b) found, for example, that it is very common practice to wash vegetables before consumption as raw salad. Although the reasons did not always reveal any understanding of pathogens and possible disease transmission, the fact that people adopted a washing behavior can be considered a significant milestone on which a local food safety campaign could build. While such post-harvest operations might not fully remove foodborne pathogens from leafy vegetables and herbs, they remain key steps complementing other options for risk
reduction (FAO/WHO, 2008). Given the basic need for food safety, a key pillar of any intervention will be creating awareness training and education.

NOTE

1. The situation is different for chemicals, especially heavy metals. Also some crops, like cucumbers or carrots, are able to absorb smaller organic chemicals, like chlorobenzenes and polycyclic aromatic hydrocarbons (Collins et al., 2006).
PROCEDURE OF PROTECTION (ALOP)
Level of protection deemed appropriate by the country establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory (WTO, 1995).

Food-Safety Objective (FSO)
The maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP) (CAC, 2004).

Health-based targets
Health-based targets are set by national authorities as a defined level of health protection for a given exposure. This can be based on a measure of disease or the absence of a specific disease related to that exposure (WHO 2004, 2006).

Control measures (CM)
Any action and activity that can be used to prevent or eliminate a food-safety hazard or to reduce it to an acceptable level (it can be microbiological specifications, guidelines on pathogen control, hygiene codes, microbiological criteria, specific information, e.g. labelling, training, education, and others (ICMSF, 2002).

Multiple-barrier approach
Protection against contaminants occurs at each step along the water to food pathway, beginning at the wastewater source, continuing at the treatment facility and extending through the farm and market chain to the kitchen where the food is prepared and eventually served (WHO, 2006; modified).

Figure 6.1 Terms and definitions for the key risk concepts in risk-based food control
Figure 6.2. Multiple-barrier approach in the wastewater food chain where treatment alone is an insufficient pathogen barrier. Source: Based on the HACCP concept, IWMI (unpublished)
There are many challenges in the detection and removal of pathogenic threats which demand some notes of caution when it comes to recommendations for risk reduction. A few are mentioned here:

- **Test conditions**: studies that have examined the survival of foodborne pathogens on plants have been mostly conducted under experimental conditions (Aruscavage et al., 2008; Jablasone et al., 2005; Stine et al., 2005). Serious limitations to the extrapolation of these experiments to real-life situations include the large initial inoculums often used and the unnatural (i.e. greenhouse/laboratory) conditions in which the plants are grown.

- **Indicator quality**: a general challenge is the use of indicator microorganisms. The detection of specific pathogens such as *Shigella* spp., *E. coli* O157:H7 or *Salmonella* spp. (see Box 12.1) is both expensive and time-consuming. Therefore, many researchers, especially in developing countries, measure thermotolerant coliform contamination frequency and magnitude as a surrogate of pathogen survival and vegetable safety (Ailes et al., 2008). However, the use of coliforms as indicators of pathogen contamination is debatable as many coliforms are naturally present in the environment and on plant surfaces and therefore their presence might not indicate recent pathogen contamination. Moreover, this group of organisms may not exhibit the same survival or attachment behaviour as the pathogens. This is particularly important when considering or assessing their removal (Lic et al., 2008).

- **Tracing contamination**: studies trying to trace the source of contamination often tend to compare independent sample sets taken, for example, at the farm gate and in markets (e.g. Armah-Klemesu et al., 1998). However, where markets receive their produce from different farms, it will require significant efforts and sample numbers to confirm the origin of any analysed difference in coliform counts. Amoah et al. (2007a) tried to bypass this problem by following vegetables from various farms – using wastewater or tap water for irrigation – to the final retail points. In this way, it was possible to identify the crucial points at which most contamination occurred in the farm to fork chain of activities.

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**Figure 6.3. Methodological challenges**
Figure 6.4. Types of disinfectants used according to the category of restaurants in Cotonou, Benin

Source: Amoah et al. (2007b)
Surface treatments with sanitizers may substantially reduce surface contamination but are significantly less effective in reducing microbial populations that have been internalized in produce (Pao and Davis, 1999). Zhuang and Beuchat (1996) demonstrated that a 15 per cent solution of trisodium phosphate completely inactivated *Salmonella* on the surface of tomatoes while only resulting in a 2 log reduction of internal populations. Moreover, some pathogens, including bacteria and some viruses, adhere to fruits and vegetables in such a fashion that they cannot be easily removed or killed with conventional washing and disinfection procedures. The exact mechanisms are not yet fully understood.

In addition to microbial contamination, washing vegetables can effectively also reduce levels of pesticide contamination. Special care is, however, required for hydrophobic pesticides which cannot easily be removed with water, unless soap is used. For some fruits and vegetables, such as tomatoes, it is best to remove the skin when boiling cannot eliminate the threat. Cocking vegetables can be contra-effective when the melting point of the pesticide is over 100°C, like in the case of Lindane analysed on tomatoes in Ghana. In this case, the tomato skin cracks when boiled and the pesticide can enter the fruit body (Obuobie et al., 2006). Amoah et al. (2006) compared the general threat of microbial and pesticide contamination of green vegetables in Ghana’s urban markets.

Figure 6.5. Limitations in view of internalized pathogens and pesticides
Supported by the Knowledge Sharing in Research (KSinR) project of the Consultative Group on International Agricultural Research (CGIAR), alternative methods of awareness creation and education on wastewater irrigation and food safety were tried in Ghana. Instead of conventional training events, farmers, food caterers, market women, retailers and representatives from authorities met in their city for an urban road trip along the contamination pathway.

The participants were taken in a bus to one of their typical urban vegetable production sites with wastewater irrigation. From there the group toured wholesale and retail markets until they reached typical street-food restaurants serving the same vegetables that they had followed from the farm. At each of the stops, farmers, vendors or kitchen staff demonstrated common and locally fitting improved practices for health-risk reduction. Participants were encouraged to ask questions and discussed possible incentives for behaviour-change at each stop along the value chain.

The road trip was supported by the visualization of the invisible threat of microbiological hazards through the use of agar plates inoculated either with wastewater (showing growing bacterial colonies) or piped water (no bacterial colonies). The main learning objectives were for:

- participants to be aware of the presence of invisible risks moving from farm to table;
- participants to understand the concept of a multiple-barrier approach with joint responsibility for effective health-risk reduction;
- authorities to appreciate and support efforts of main stakeholders to contribute to solutions.

Source: Amoah et al. (2009)

Figure 6.6. Road shows
Table 6.1: Factors affecting pathogen survival in the environment

<table>
<thead>
<tr>
<th>Factor</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humidity/Precipitation</td>
<td>Humid environments favour pathogen survival. Dry environments facilitate pathogen die-off. Rainfall can result in splashing of contaminated soil on crops.</td>
</tr>
<tr>
<td>Temperature</td>
<td>Most important factors in pathogen die-off. The impact of temperature varies for different pathogens. High temperatures lead to rapid die-off, normal temperatures lead to prolonged survival.</td>
</tr>
<tr>
<td>Acidity/Alkalinity (pH)</td>
<td>Some viruses survive longer in more acid, i.e. lower pH soils, while alkaline soils are associated with more rapid die-off of viruses. Neutral to slightly alkaline soils favour bacterial survival.</td>
</tr>
<tr>
<td>Sunlight (UV radiation)</td>
<td>Direct sunlight leads to rapid pathogen inactivation through desiccation and exposure to UV radiation.</td>
</tr>
<tr>
<td>Foliage/plant type</td>
<td>Certain vegetables have sticky surfaces (e.g. zucchini) or can absorb pathogens from the environment (e.g. lettuce, sprouts) leading to prolonged survival. Root crops are more prone to contamination and facilitate pathogen survival.</td>
</tr>
<tr>
<td>Competition with native flora and fauna</td>
<td>Antagonistic effects from bacteria or algae may enhance die-off.; Bacteria may be preyed upon by protozoa.</td>
</tr>
</tbody>
</table>

Source: Strauss (1985); modified
Table 6.2: Survival times of selected excreted pathogens in soil and on crop surfaces at 20-30°C

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Survival time in Soil</th>
<th>Survival time on crops</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viruses:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enteroviruses&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;100 but usually &lt;20 days</td>
<td>&lt;60 but usually &lt;15 days</td>
</tr>
<tr>
<td>Bacteria:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faecal coliform</td>
<td>&lt;70 but usually &lt;20 days</td>
<td>&lt;30 but usually &lt;15 days</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>&lt;70 but usually &lt;20 days</td>
<td>&lt;30 but usually &lt;15 days</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>&lt;20 but usually &lt;10 days</td>
<td>&lt;5 but usually &lt;2 days</td>
</tr>
<tr>
<td>Protozoa:</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em> cysts</td>
<td>&lt;20 but usually &lt;10 days</td>
<td>&lt;10 but usually &lt;2 days</td>
</tr>
<tr>
<td>Helminths:</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ascaris lumbricoides</em> eggs</td>
<td>Many months</td>
<td>&lt;60 but usually &lt;30 days</td>
</tr>
<tr>
<td>Hookworm larvae</td>
<td>&lt;90 but usually &lt;30 days</td>
<td>&lt;30 but usually &lt;10 days</td>
</tr>
<tr>
<td><em>Taenia saginata</em> eggs</td>
<td>Many months</td>
<td>&lt;60 but usually &lt;30 days</td>
</tr>
<tr>
<td><em>Trichuris trichiura</em> eggs</td>
<td>Many months</td>
<td>&lt;60 but usually &lt;30 days</td>
</tr>
</tbody>
</table>

<sup>a</sup>Includes polio-, echo-, and coxsackie-viruses.

Source: Feachem et al. (1983)
References


Chapter 7 - Conclusions

The scientific literature in microbial food safety of fresh vegetables has grown considerably over the past two decades; however, data necessary to support the development of evidence-informed recommendations, effective interventions and sound policies are still missing.

Several research areas that contain consistent data for further pooled analysis were highlighted. Almost 80% of identified published data in microbial safety of leafy greens was not suitable for policy and decision making due to major deficiencies in the conduct and/or reporting of primary research in this area. Main knowledge gaps and future research needs were identified, and several knowledge gaps were addressed in this work.

Chlorine washes are most commonly used by fresh vegetable processing industry and their primary role is to maintain the sanitary conditions of wash water and prevent cross-contamination, while the reduction of microbial contamination is their expected outcome. We demonstrated that washing of leafy greens does not result in reduction of bacterial counts in large-scale commercial conditions. Minimal processing of spinach resulted in either no change or increased coliform contamination in most (>80%) lots of processed spinach. Washing of fresh-cut lettuce was also ineffective, as the bacterial
counts in packed product increased to pre-washing levels. However, minimal processing had an overall positive effect. Outer leaf removal as a step in large-scale lettuce processing was associated with the decreased concentrations of contamination indicators. These findings are of relevance to development of improved interventions for food safety and shelf-life extension in the industry. In addition, this information can be utilized for determination of critical points for the required HACCP-based food safety systems.

The implementation of the system approaches is a major challenge in the developing regions where the collective organization, food safety education, risk awareness, and resources to undertake intervention studies are often lacking. Our synthesis of multi-barrier interventions in post-harvest chain of vegetable production moves the knowledge acquired through research to actual application in practical settings and circumstances of settings and circumstances of developing countries.
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