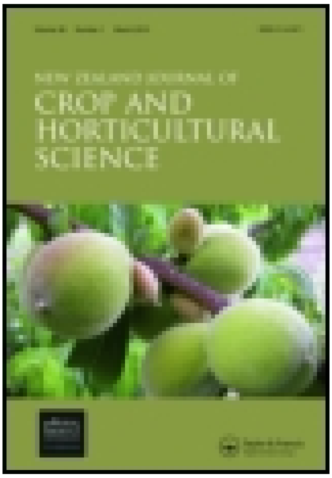

New Zealand Journal of Crop and Horticultural Science
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tnzc20

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To cite this article: G Ávila-Quezada , E Sánchez , AA Gardea-Béjar & E Acedo-Félix (2010) Salmonella spp. and Escherichia coli: survival and growth in plant tissue, New Zealand Journal of Crop and Horticultural Science, 38:2, 47-55, DOI: 10.1080/01140671003767834

To link to this article: http://dx.doi.org/10.1080/01140671003767834

New Zealand Journal of Crop and Horticultural Science
Vol. 38, No. 2, June 2010, 47–55

Salmonella spp. and *Escherichia coli*: survival and growth in plant tissue

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(*Received 20 July 2009; final version received 16 March 2009*)

This review presents information on disease outbreaks in human populations linked to *Salmonella* spp. and *Escherichia coli* O157:H7 associated with the consumption of fresh produce. It focuses on the processes of bacterial internalization and survival in non-host plant tissues and the role of biofilms in bacterial persistence. Research to identify the microbial sources contaminating fruits and vegetables and their persistence is urgently required. Internalization of human pathogenic bacteria in plant tissues is reported for various fruits. Under certain circumstances these can enter the plant via roots or seeds, with subsequent survival and translocation within the plant. Research should continue on the relationships between human pathogens and plants, and treatments should be developed to minimize pathogen presence on the surface of raw produce and to prevent their internalization.

Keywords: internal plant tissues; bacterial internalization; food safety; outbreaks

Introduction

In recent decades, there has been an increase in reports of produce-associated disease outbreaks in humans. These may be related to changes in consumer food preference, in food production practices and in the emergence of new food-borne pathogens. Investigation of food-borne outbreaks has proved a difficult task. Bacteria such as *Escherichia coli* O157:H7 (*Ec*O157) (Besser et al. 1993; Ackers et al. 1998) and *Salmonella* (Isaacs et al. 2005; Singh et al. 2006) are commonly involved in such outbreaks.

The produce items most frequently implicated in *Salmonella* and *Ec*O157 outbreaks are salads, sprouts, melons, lettuces, tomatoes, precut celery, apple and orange juices, broccoli, berries, squash, cantaloupes, alfalfa sprouts and mixed fruits (Duffell et al. 2003; Sivapalasingam et al. 2004; Söderström et al. 2005; Mukherjee et al. 2006). During the past 10 to 15 years, *Salmonella* and *Ec*O157 have been the two most common etiological agents responsible for produce-related outbreaks in the US (Mukherjee et al. 2006). To cause human disease, pathogens contaminating fruits and vegetables must survive until consumed. It seems that lettuce is the food most susceptible to contamination by pathogens that affect humans.

Fluid leakage from lettuce tissue as a result of minimal processing may provide sufficient nutrients to support the growth of *Ec*O157 even in the presence of high numbers of other naturally existing microorganisms (Beuchat 1999).

Salmonella spp. and *Ec* can enter, invade and migrate in plant tissues such as seeds, fruits, leaves, roots and stems, as determined by fluorescence microscopy and by laser

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ISSN 0114-0671 print/ISSN 1175-8783 online

DOI: 10.1080/01140671003767834
http://www.informaworld.com

scanning confocal microscopy (LSCM) (Brandl & Mandrell 2002; Solomon et al. 2002). The recognition mechanism between bacteria and plants remains unknown. In recent years, there have been important research advances in bacterial communities, such as biofilm formation and its role in disease production. There have been concerted efforts to develop a definition of this phenomenon and Costerton et al. (1999) have defined a bacterial biofilm as a structured community of bacterial cells enclosed in a self-produced polymeric matrix that is adherent to an inert or a living surface. To obtain a fuller understanding of the mechanisms involved in these phenomena and to allow more appropriate research planning, the aim of this review is to provide an update on information on this 'symbiotic relationship' between *Salmonella* spp. and *Ec* and common food plants.

Outbreaks associated with *Escherichia coli* O157:H7

Initial outbreaks were found on meats. *Ec*O157 was first identified as a possible human pathogen in 1975 in a sick patient; this outbreak was associated with the consumption of ground beef (Doyle et al. 2006). The most likely natural carriers are wildlife; ruminants have been identified as the major *Ec*O157 reservoir, while domestic cattle are the most important source for human infections. Other ruminants known to carry these bacteria include sheep, goats and deer (Doyle et al. 2006).

Outbreaks of *Ec*O157 infections in the US have also been associated with the consumption of fruits and vegetables (Besser et al. 1993; CDC 1995; Ferguson et al. 2005; CDC 2006a; CDC 2006b; Reiss et al. 2006). In the case of an *Ec*O157 multi-state outbreak, infections from fresh spinach were reported from 26 states in the US (CDC 2006a; CDC 2006b). The Food and Drug Administration (FDA) and the California Department of Health Services (CDHS) carried out an extensive investigation into the causes of an *Ec*O157 outbreak associated with contaminated 'Dole' brand baby spinach, which resulted in 205 confirmed illnesses and three deaths. The probe focused initially on the processing and packaging plant of Natural Selection Foods, in San Juan Bautista, California, where the contaminated products were processed. The next focus of the inquiry was the source of the spinach from 13 bags containing *Ec*O157 isolates that had been collected nationwide from sick customers. Using the bar codes on bags and performing DNA fingerprinting on the bacteria from these bags, researchers were able to match environmental samples of *Ec*O157 from one field to the strain that had caused the outbreak. Potential environmental risk factors for field contamination included the presence of wild pigs, the proximity of irrigation wells used to grow produce for ready-to-eat packaging and surface waterways exposed to cattle and wildlife faeces. Because of the many ways that *Ec*O157 can be transferred, the precise means by which the bacteria spread to the spinach remains unknown (FDA 2006). A final report identified the contamination source as a cattle operation nearby.

A similar situation occurred in Japan in 1996, it was the largest known *Ec*O157 outbreak involving 11,826 cases and 12 deaths. Infection appeared to be transmitted through the consumption of raw radish sprouts served in lunches. However, the source of the radish contamination remains unknown (Michino et al. 1999).

The dose response for infection by this pathogen is low. Epidemiological studies of food-borne outbreaks indicate that fewer than 40 viable cells of *Ec*O157 can cause illness in some people (Teunis et al. 2004; Strachan et al. 2005).

Contamination and persistence of *Escherichia coli*

Ishii et al. (2006) reported that the population of 'naturalized' *Ec* in soil can reach 10^5 CFU/g in northern temperate areas during the warmest months. This suggests that some of the *Ec* positive soil samples could have been contaminated with indigenous soil strains and that their presence may not necessarily indicate exposure of produce to faecal material. Studies on organic produce in the UK usually do not reveal contamination with either *Salmonella* or *Ec*O157 (McMahon & Wilson 2001; Sagoo et al. 2001). In a survey in Norway, these two bacteria were not detected in 179 organically grown lettuce samples collected from 12 growers (Loncarevic et al. 2005). Also, neither *Ec*O157 nor *Salmonella* spp. were detected in a study on fruit, vegetables and irrigation water in Mexico (Ávila-Quezada et al. 2008).

In a study on smoked 'chipotle' pepper carried out in an open-oven (outdoors), *Ec* was found to be absent in the 48 analysed samples. This absence was attributed to the action of some compounds in the wood smoke, which may control microorganisms through some sort of biocidal action (Ávila-Quezada et al. 2009).

Although the mechanism of inoculation with *Ec*O157 is frequently unknown, washing water is thought to be the most likely source for lettuce contamination (Rangel et al. 2005). Studies on cattle have shown that two or three per 1000 animals carry *Ec*O157 (Hancock et al. 1994) and this strain has been shown to survive in cattle faeces for up to 70 days (Wang et al. 1996). Lettuce could be a biological vehicle, since growth studies demonstrate that surface populations of *Ec*O157 increase for up to 14 days at both 12 and 21°C (Abdul-Raouf et al. 1993).

Field persistence of *Ec*O157 is variable and affected by numerous factors. Recent studies indicate that *Ec*O157 survives in the field for about four to eight weeks (Johannessen et al. 2005). This is the reason for recommending a minimum of 120 days between manure application and harvest of plant produce. Some studies have concluded that *Ec*O157 survival improves at cool temperatures, in clay soils and in close association with roots (Gagliardi & Karns 2002; Ingham et al. 2005). A recent study reported that *Ec*O157 can be isolated from carrots 168 days after application of contaminated manure (Islam et al. 2005).

*Ec*O157 transmission from manure-contaminated soil and irrigation water to lettuce plants was demonstrated using laser scanning confocal microscopy and epifluorescence microscopy, and the recovery of viable cells from the inner tissues of plants (Solomon et al. 2002), as well as green fluorescent protein (GFP) techniques to detect internalization of *Ec*O157 and *Salmonella enterica* (Franz et al. 2006), because isolation alone does not give information on the exact location of the bacterial cells.

Outbreaks associated with *Salmonella* spp.

Outbreaks of *Salmonella* infections in people in the US have been traced to many crops, fruits and vegetables (Gayler et al. 1955; Blostein 1993; CDC 1993; Mahon et al. 1997).

There are many cases involving *Salmonella* in food-borne outbreaks, but two cases demand special attention. An international outbreak of *Salmonella* Stanley across 23 states in the US and Finland was linked to the consumption of alfalfa sprouts in 1995 (Mahon et al. 1997). The sprouts were traced back to nine growers and one distributor, a Dutch shipper. A year later (1996), an outbreak of *Salmonella* Newport was associated with sprouts in Oregon and British Columbia and traced to seed from the same Dutch shipper (Van Beneden et al. 1999). Although these were different strains, it is likely that the ship was the source of contamination of *Salmonella* spp.

Sources of *Salmonella* spp. contamination and their persistence

Salmonella can persist in the free environment for extended periods. These bacteria were found to survive in soil samples for 203 to 231 days (Islam et al. 2004). *Salmonella* survival was greatest in soil treated with poultry compost and least in soil containing alkaline-pH-stabilized dairy cattle manure compost. Also, in the same study, survival profiles of *Salmonella* on vegetables and in soil samples contaminated by irrigation water were similar to those observed when contamination occurred through compost. Hence, both contaminated manure compost and contaminated irrigation water can have important roles in contaminating soil and root vegetables with *Salmonella* for several months (Islam et al. 2004).

There is also a possibility that a kind of symbiosis exists on plant produce between the plant and human pathogens. Wells and Butterfield (1997) demonstrated that *Salmonella* grew more quickly on potato, carrot and green pepper when co-cultured with *Erwinia carotovora*.

Bacteria can survive for extended periods on fresh produce as has been reported for *Salmonella* spp. on melons kept for 6 h at 22–27°C (Escartin et al. 1989) and on alfalfa sprouts for 10 days at 5°C (Jaquette et al. 1996); under certain favourable conditions this pathogen may also grow in tomatoes at 20°C (Zhuang et al. 1995) and in melons at 23°C (Escartin et al. 1989).

Biofilms

Many bacteria exude exopolysaccharides to form biofilms, which adhere to surfaces from where they can be disseminated to foods. As revealed by various microscopic techniques, the microstructures of biofilms are highly complex and consist of many symbiotic organisms, some of which are human pathogens. In their natural environment, microorganisms can survive many changes, even though they continually compete among themselves. In this environment, the cells must adapt to the surrounding conditions or they die. Most cells survive by adhering to a surface, after that they lie under a polysaccharide protective layer. In time, this layer becomes a biofilm where different microorganisms co-exist. In the biofilm, the microorganisms develop their own microenvironment in which they survive. Besides protecting them from a hostile environment, the biofilm acts as a trap for acquiring nutrients. Biofilm formation causes problems in the food industry causing energy loss, reducing flow and increasing heat transmission or blocking filter membrane pores. Flow variations or cleaning procedures can cause pieces of this biofilm to detach and to spread in the process flow (Veno 1999).

Nowadays, we know that in Gram-negative bacteria (*Pseudomonas aeruginosa*, *Vibrio cholerae*, *Escherichia coli*, *Salmonella enterica*), flagella, fimbriae types I, IV and curlie, are important bacterial structures for the primary stages of adherence. Motility appears to help the bacteria to reach stable surfaces and to counteract their hydrophobic repulsions. However, although motility helps the process, it does not seem to be an essential requirement for adhesion. In later stages, the bacteria secrete exopolysaccharide, which comprises the biofilm matrix and forms a mushroom-like structure, which shows the presence of channels. The composition of the exopolysaccharide is different for each bacterial species—examples include alginates in *P. aeruginosa*, cellulose in *S. typhimurium*, an exopolysaccharide rich in glucose and galactose in *V. cholerae*, and poly-N-acetylglucosamine in *S. aureus* (Costerton et al. 1999; Nobile & Mitchell 2007).

Furthermore, recent studies have shown that a single bacterial species, depending on the environmental conditions in which it is found, can produce different exopolysaccharides as matrix components of a biofilm (O'Toole et al. 2000). *E. coli* O157:H7 and *Salmonella* spp. have been reported to have the potential to persist even on inert surfaces, such as stainless steel, dental plaque and glass. It has been hypothesized that this capability may be implicated in cross contamination, since supermarket meat grinders have been suspected to be involved in outbreaks of both bacteria. The ability of biofilm formation does not appear to be restricted to any particular group of microorganisms and today it is considered that under a suitable environment almost all microorganisms are able to form some sort of biofilm (Nobile & Mitchell 2007).

Biofilms on plants

Biofilms or aggregates on plant surfaces are colonies of cells in an exopolymer matrix that can protect individuals from desiccation or bactericidal agents, acting also as pools for genetic material exchange (Morris & Monier 2003). As an example, biofilms have been found composed of rod-shaped bacteria on young alfalfa sprouts (Fett 2000).

In a recent study, the adhesion and persistence of *Salmonella typhimurium* and its biofilm-deficient isogenic mutant were compared on parsley. The authors concluded that the biofilm matrix of *Salmonella* is not likely to play a significant role in the initial adhesion and survival after disinfection. After a week of storage, the biofilm-producing strain survived chlorination significantly better than the biofilm-deficient mutant. However, the recovery of the mutant was still high, indicating that although the biofilm matrix has a role in the persistence of *Salmonella* after chlorination treatment of parsley, this was not the single most important mechanism, and other mechanisms may provide protection, probably the ability to penetrate the plant tissue or preexisting biofilms, or the production of polysaccharides other than cellulose (Lapidot et al. 2006).

Internalization of bacteria into deeper plant tissues

Pathogen internalization has been reported in non-bruised tomatoes (Zhuang et al. 1995), apples (Buchanan et al. 1999) and oranges (FDA 1999). There is the possibility of interactions between the plant and the human pathogen in entry to the plant. Recent studies demonstrated that human bacterial pathogens establish a close relationship with the underground parts of the plant as well (Gagliardi & Karns 2002; Natvig et al. 2002; Ingham et al. 2005), penetrating to the internal tissues through the roots (Solomon et al. 2002), and seeds (Islam et al. 2004; Natvig et al. 2002) for further translocation and survival in the edible aerial plant tissues (Solomon et al. 2002).

Some studies demonstrate that the epidermis does not prevent the internal contamination of produce, because bacteria from contaminated water have been found to enter fruits and vegetables under certain conditions. The internalization is often due to a non-controlled wash or a fast pre-cooling treatment applied to warm produce. When warm fruits or vegetables are immersed in cold water, the resulting pressure difference between the produce core and the water allows pathogens in the water to enter the core, almost always through the stem-surrounding area (Bartz & Showalter 1981). In apples, the entry is more commonly from the other end of the fruit (from the flower end)—in the so-called ‘open-calyx’ cultivars. Also, via the necrotic sepal tips with the pathogen migrating down the xylem to form nodules in the flesh.

In addition, bruises increase the potential for contaminated water internalization in fruits during washing. Internalization of bacteria in processing water has been shown in apples (Foster & Hall 1990) and radish sprouts (Itoh et al. 1998). The water used for sprouting could be another source of contamination; research has demonstrated that when roots of fully developed radish sprouts are immersed in water containing *Ec*O157, the pathogen is found throughout the edible portion of the sprout, even after surface decontamination. This study suggests that inner tissues of sprout can become contaminated by bacteria (Itoh et al. 1998).

E. coli can penetrate via the stomata and cut edges of lettuce leaves (Seo & Frank 1999), which results in their protection from superficial chlorine treatment (Tekeuchi & Frank 2000). Some results have suggested that *Salmonella* can enter fruits, and probably other plant parts, through abrasions, where they may persist (Guo et al. 2001; Guo et al. 2002).

The ability of two strains of *Salmonella* to form biofilms was studied by Annous et al. (2005). In this study, biofilms occurred rapidly following the introduction of cells onto the melon surface. Fibrillar material was visible after just two hours, and cells were embedded in an extracellular polymeric material after 24 hours. These results indicate that a human pathogen is capable of forming a biofilm on plant tissues and that biofilm formation may be responsible for the increased recalcitrance of attached bacteria to aqueous superficial sanitizers (Annous et al. 2005).

In some cases, tomatoes involved in multistate outbreaks of *Salmonella* infections have been traced to packing houses where tomatoes were washed in a common water bath (Hedberg et al. 1999). Dip-washing tomatoes may result in the diffusion of water to the fruit interior. If the wash water is contaminated or if a proper disinfection procedure is not followed, pathogens may also contaminate the product’s internal tissues (Ibarra-Sanchez et al. 2004). Or if the pathogens are on the surface only, these can be transferred to the flesh during handling or cutting (Lin & Wei 1997), where they can survive and even multiply (Bartz 1988).

Salmonella spp. can survive and grow on the surface of mature, intact tomatoes at ambient temperature (Zhuang et al. 1995). Many of the patients involved in the outbreak infection of *Salmonella* Montevideo in Illinois (CDC 1993) had washed the tomatoes and had removed the stem core, so it is probable that *Salmonella* came from inside the fruits (Lin & Wei 1997). As mentioned earlier, internalization may result from a pressure differential caused by very different temperatures between produce and wash, in other words, it is required that the fruit be warmer than the wash water (not the other way around), then the internal air contracts with cooling and the internal pressure is lowered, so drawing in air and water from outside.

Concentrations of 6.55×10^5 CFU/g of *Salmonella typhimurium* can invade seeds and undergo a 3.5 log increase during germination. In this study, the large concentration of bacteria found in plants grown in sterile soil inoculated with *Salmonella typhimurium*, even after 45 days of growth, could be due to a lack of competition. In the same study, more apical leaves had significantly higher concentrations of *Salmonella*. In this study, this bacterium reached a height of 20 cm inside the stem of mung bean plants (Singh et al. 2005). In other studies, *Salmonella* migrated short distances, 9–10 cm, after entry into plants through abrasions (Guo et al. 2001).

The use of hyperchlorinated water reduces, but does not eliminate, the *Salmonella* count on external plant surfaces and, of course, has an even lesser effect on internal populations of this bacterium (Zhuang et al. 1995). Disinfection treatments are designed to reduce the incidence of microorganisms on the surface of plant produce, but if the microorganisms are located deep within the product, then no contact occurs between them and the disinfectant. Because of all of the above, the presence of *Salmonella* in cultivated soils represents a serious health risk to consumers of fresh produce and the bad press resulting from a disease outbreak traced to this source can cause serious economic damage to growers.

Conclusions

Compost and irrigation water containing raw sewage or improperly treated effluents from sewage treatment plants may contain pathogenic bacteria. Produce can be contaminated due to internalization of those pathogens, both through the root system and skin or stem scars. *In vitro* evidence suggests that pathogens can be incorporated into fresh produce by these means, but this has not been demonstrated in the field in real situations.

Some epiphytic or plant pathogenic bacteria can assist *E. coli* and *Salmonella* survival by modifying the host tissue and supplying a suitable environmental niche. Many bacteria produce exopolysaccharides to form biofilms, which help them to adhere to plant or inert surfaces from where they can be disseminated to foods.

Human pathogens can also use polysaccharides from plants to survive until plant tissues develop protective coatings in vascular vessels or develop some chemical moiety or activate some biological phenomenon acting locally for the elimination of *Salmonella* and *E. coli*.

E. coli and *Salmonella* can survive in both soil and in plants. Occasionally, conditions arise leading to food produce contamination. Survival of these pathogens is controlled by complex interactions with plants. Understanding such interactions will assist in the development of new strategies to improve food safety. To explicitly define and recognize the interactions and translocation of human pathogenic bacteria in plants, detailed studies involving molecular tracking are essential.

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