ORIGINAL ARTICLE

Evidence of an antilisterial factor induced by wounding of iceberg lettuce tissues

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Abstract

Aims: To examine the influence of wound-associated reactions in cut iceberg lettuce (*Lactuca sativa* L.) tissues on the fate of *Listeria monocytogenes*.

Methods and Results: Aqueous extracts prepared from shredded iceberg lettuce before and after storage in high oxygen permeability film were inoculated with *L. monocytogenes*. *Listeria monocytogenes* grew in extracts prepared from fresh lettuce. In contrast, inhibition ranging from arrested growth to a decline in cell viability was observed in extracts prepared from samples stored for 1–3 days. Similar behaviour was evident in lettuce shreds inoculated with10^5 CFU g^{-1} *L. monocytogenes* immediately after processing or after 3 days in storage. Heat treatment of the cut tissues at 47°C for 3 min before storage diminished the inhibitory effect.

Conclusions: The results provided evidence that an antilisterial factor or factors are released by wounded iceberg lettuce tissues. Antilisterial activity was mitigated by heat treatment of the lettuce.

Significance and Impact of Study: This study indicates that intrinsic factors associated with plant metabolism could play a significant role in the ecology of human pathogens in packaged horticultural products.

Introduction

Although it is well established that the psychrothrophic foodborne pathogen *Listeria monocytogenes* can grow in refrigerated, packaged fresh-cut iceberg lettuce, behaviour of the species in this ecosystem remains poorly understood. Some reports describe growth stimulation postulated to result from alterations in the size and possibly the composition of the spoilage microflora in response to selective pressures induced by modified atmospheres (Steinbruegge *et al.* 1988; Farber *et al.* 1998; Jacxsens *et al.* 1999). Conflicting observations on the effects of modified atmospheres are also found in the scientific literature however. Growth patterns for *L. monocytogenes* inoculated onto lettuce stored in air and under anoxic atmospheres were identical in a study by Beuchat and Brackett (1990). Furthermore, attempts to verify the competition hypothesis by co-inoculation with variable populations of native micro-organisms have been inconclusive. For example, Francis and O'Beirne (1997) found that survival *L. innocua* was unaffected by background populations ranging from 10^3 to 10^7 CFU g^{-1}.

The maintenance of oxygen-depleted atmospheres and low storage temperatures reduces enzymatic browning in packaged lettuce. Despite these measures, shelf-life is restricted by the appearance of discolourations and additional means to alleviate the problem are under investigation. A potential solution involves the application of mild heat treatments or heat shocks before packaging (Delaquis *et al.* 1999, 2002; Loaiza-Velarde *et al.* 1997; Loaiza-Velarde and Saltveit 2001; Saltveit 2000). Temperatures between 47 and 50°C for 90–180 s delay the onset of browning by several days in refrigerated product. In addition, such temperatures are lethal to micro-organisms associated with the plant surface and population reductions 1–2 log CFU g^{-1} lettuce higher than those achieved with conventional washes in cold chlorinated water have been reported (Delaquis *et al.* 1999). Unfortunately, the

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perceived advantages of heat treatments are tempered by evidence of accelerated microbial growth during subsequent refrigerated storage. Faster development of the spoilage microflora has been observed and inoculation with *L. monocytogenes* confirmed that growth is enhanced by prior heat treatment of the lettuce (Li *et al.* 2002; Delaquis *et al.* 2002).

Tissue wounding is unavoidable during processing of lettuce into fresh-cut products. Wounding induces numerous physiological alterations including elevated respiration rates, ethylene synthesis, oxidative browning, wound healing reactions, secondary metabolite synthesis and water loss (Brecht 1995; Cantos *et al.* 2001; Kang and Saltveit 2003). The appearance of browning is associated with phenylpropanoid metabolism, a series of reactions that can, with the intervention of polyphenol oxidase, lead to the formation and accumulation of brown phenolic compounds (Camm and Towers 1973). *De novo* synthesis of phenylalanine ammonia lyase (PAL) under aerobic conditions and PAL catalyzed deamination of L-phenylalanine to trans-cinnamic acid and ammonia is the first step in a series of reactions referred to as the phenylpropanoid pathway. Heat shock (Fukumoto *et al.* 2002) and modified atmospheres (López-Gálvez *et al.* 1996) both inhibit PAL activity and the accumulation of phenylpropanoid metabolites in cut lettuce tissues. The role of these reactions in the ecology of microbial spoilage or the fate of foodborne pathogens in plant foods is not known. Enhanced growth of *L. monocytogenes* in cut lettuce subjected to heat shocks or storage under modified atmospheres hints that such treatments may weaken intrinsic barriers to growth. Moreover, the ability to modulate wound-associated reactions by heat shock may provide a useful experimental tool for the study of putative interactions between plant tissues and colonizing micro-organisms. This approach was exploited in the present investigation on the interaction between *L. monocytogenes* and lettuce tissues.

Materials and methods

Preparation of lettuce shreds and lettuce extracts

Experiments were carried with shreds or aqueous tissue extracts prepared from whole lettuce (*Lactuca sativa* L. type Salinas) heads obtained from a local retailer. The latter were grown in California and were delivered to the retail outlet approx. 3 days after harvest. Outer and damaged leaves were discarded and shreds (3 × 20 mm) were cut from both photosynthetic and vascular tissues using a stainless steel knife. Where necessary, the shreds were packed in bags (30 × 17 cm) made of PD941 film (Cryovac, Mississauga, ON, Canada; oxygen transmission rate 16 544 ml O_2 m^{-2} 24 h^{-1}) that were closed with 2-mm wide seals using a SwissVac vacuum sealer (SwissVac, Luzern, Switzerland). Aqueous extracts were prepared by placing 50 g of shreds in 250-ml Erlenmeyer flasks (VWR, Edmonton, AB, Canada) with 100 ml sterile distilled water, followed by agitation on an orbital shaker at 125 rpm for 1 h at room temperature. The extracts were then filtered through Whatman no. 4 filter paper (Whatman, Florham Park, NJ, USA), spun at 10 000 ***g*** for 30 min at 4°C in a centrifuge (RC 5B Plus; Sorvall, Thermo Electron, Asheville, NC, USA) and sterilized by passing through 0·22 μm membranes (Supor 200; Millipore, Billerica, MA, USA).

Microbiological methods

Experiments were carried out with *L. monocytogenes* strain CFAR 92 (graciously provided by Dr R. McKellar, AAFC, Guelph, ON, Canada). The strain was originally isolated from chicken wiener and is sensitive to the inhibitory effects of phenolic acids (Wen *et al.* 2003). Stock cultures were maintained at 4°C on Trypticase Soy Agar (BBL; BD Biosciences, Mississauga, ON, Canada) amended with 5 g l^{-1} Yeast Extract (TSYA; Oxoid, Nepean, ON, Canada). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to 10 ml Trypticase Soy Yeast Extract Broth (TSYB; BD Biosciences), followed by incubation overnight at 30°C. Inocula were prepared by centrifugation of the culture at 3220 ***g*** for 15 min at room temperature. Sterile distilled water (10 ml) was added to re-suspend the cells by gentle agitation in a laboratory mixer and the tubes were spun anew at 3220 ***g*** for 15 min. The density of the final washed cell suspension was adjusted with sterile distilled water in a spectrophotometer (620 nm) to obtain approx. 5×10^6 CFU ml^{-1}.

Cell populations in cultures or aqueous lettuce extracts were determined by spreading appropriate dilutions prepared in 0·1% (w/v) peptone onto TSYA, followed by incubation at 30°C for 48 h. Where enrichment was necessary for the detection of cell densities below the limit of detection, 10 ml of the extract were added to 100 ml TSYB. Enrichment medium fluids were spread onto PALCAM agar (Difco; BD Biosciences) after 48 h incubation at 30°C. Bacterial populations in cut lettuce were determined from homogenates prepared by pummelling 50 g lettuce with 450 ml 0·1% peptone in a Lab Stomacher (Seward, Colworth, UK). Appropriate dilutions in 0·1% peptone were spread onto Plate Count Agar (Difco) and PALCAM agar (Difco) to estimate total aerobic and *L. monocytogenes* populations respectively. Plate Count Agar plates were incubated at 30°C for 48 h in air and PALCAM plates were incubated at 30°C for 48 h in

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anaerobic jars to discourage the growth of aerobic micro-organisms. Several isolated colonies were picked from the PALCAM plates for confirmation by the latex test (*L. monocytogenes* Latex Test; Oxoid).

Fate of *L. monocytogenes* in aqueous lettuce extracts

Sterile aqueous extracts were prepared from fresh lettuce and from packaged shreds stored for 1, 2 and 3 days at 15 ± 1°C on metal shelves in a darkened incubator. One hundred millilitres of each extract were placed in 250 ml screw-capped Erlenmeyer flasks. Inoculum was added to achieve initial cell populations of approx. 10^4 CFU ml^{-1} *L. monocytogenes* and the flasks were incubated at 25 ± 1°C with agitation. Samples were periodically withdrawn to estimate population densities. Three independent trials were carried out.

Fate of *L. monocytogenes* in extracts prepared from washed and stored shreds

Lettuce shreds were prepared as before. Seven hundred grams were dipped for 3 min in a stainless steel container with 14 l of a 100 μg ml^{-1} sodium hypochlorite solution cooled to 4°C or heated to 47°C. Excess water was removed in a home salad spinner immediately after treatment and the shreds were packed and stored at 15 ± 1°C. Aqueous extracts were prepared from lettuce subjected to each treatment immediately after preparation and after 3 days in storage. Extracts inoculated with *L. monocytogenes* were incubated at 25 ± 1°C and cell populations were estimated over time.

Fate of *L. monocytogenes* in aqueous phenolic depleted lettuce extract

Phenolic compounds were removed from an aqueous extract prepared from fresh lettuce by passage through a hydrophobic Sep-Pak C18 column (Waters, Milford, MA, USA). Total phenolic content before and after passage were measured using the Folin–Ciocalteau reagent method of Singleton and Rossi (1965). Unaltered and phenolic-depleted extracts were inoculated with *L. monocytogenes* and cell populations were estimated during incubation at 25 ± 1°C.

Fate of *L. monocytogenes* in packaged lettuce shreds

Lettuce shreds were placed in film bags without further treatment and after chlorinated water washes at 4 or 47°C. One half of the packages were immediately inoculated with 10^5 CFU g^{-1} *L. monocytogenes* by direct addition of 0·5 ml of a washed cell suspension. The packages were sealed, inverted several times to mix the contents and placed in an incubator at 15 ± 1°C. The second half was stored at 15 ± 1°C for 3 days before inoculation. Inocula were added through small openings made with sterile scissors, which were closed with a handheld sealer before returning the packages to the incubator. Two packages from each treatment combination were analysed daily over 3 days to determine changes in total aerobic and *L. monocytogenes* populations. The experiment was repeated three times.

Results and discussion

The experimental strain chosen for this work grew well in aqueous extracts prepared from fresh lettuce obtained on three separate occasions (Fig. 1). In contrast, declining cell densities indicative of inhibitory activity were evident in all extracts prepared from shreds stored at 15 ± 1°C for 1, 2 or 3 days. The effect was always more pronounced in extracts made from lettuce stored for >1 day and was strongest in lettuce stored for 2 days, in two out of three trials. In both cases cell densities were below the limit of detection for the plating assay (10 CFU ml^{-1}) after 2–3 h incubation and attempts to recover viable cells after 25 h incubation were unsuccessful. These observations provided evidence that a water soluble factor or factors lethal to *L. monocytogenes* accumulated in cut lettuce tissues stored under aerobic conditions. Aqueous extracts were also prepared from cut lettuce subjected to a variety of physical treatments to determine whether the appearance of inhibitory activity was affected by heat-induced physiological alterations. As shown in Fig. 2, strong inhibition of *L. monocytogenes* was apparent from declining cell densities in extracts prepared from lettuce stored for 3 days without treatment or following a wash in cold chlorinated water, a common practice in the fresh-cut industry. The consequences of heat treatment before storage were not as clear. Effects ranged from growth inhibition without loss in cell viability (trial I), no inhibition (trial II) and extensive cell death (trial III). These apparent discrepancies were postulated to arise from variability in raw materials because of varietal, seasonal, agronomic or postharvest handling, all factors known to affect physiological reactions in plant tissues.

Evidence of a time-dependant release of inhibitory factor (s) by cut tissues invited speculation about a possible role for such reactions in the ecology of *L. monocytogenes* in stored, packaged lettuce. The fate of the species was consequently examined in shreds inoculated with 10^5 CFU g^{-1} immediately after processing or after 3 days in storage. Figure 3 shows total aerobic and *L. monocytogenes* populations after 3 days storage at 15 ± 1°C. *Listeria monocytogenes* populations remained relatively

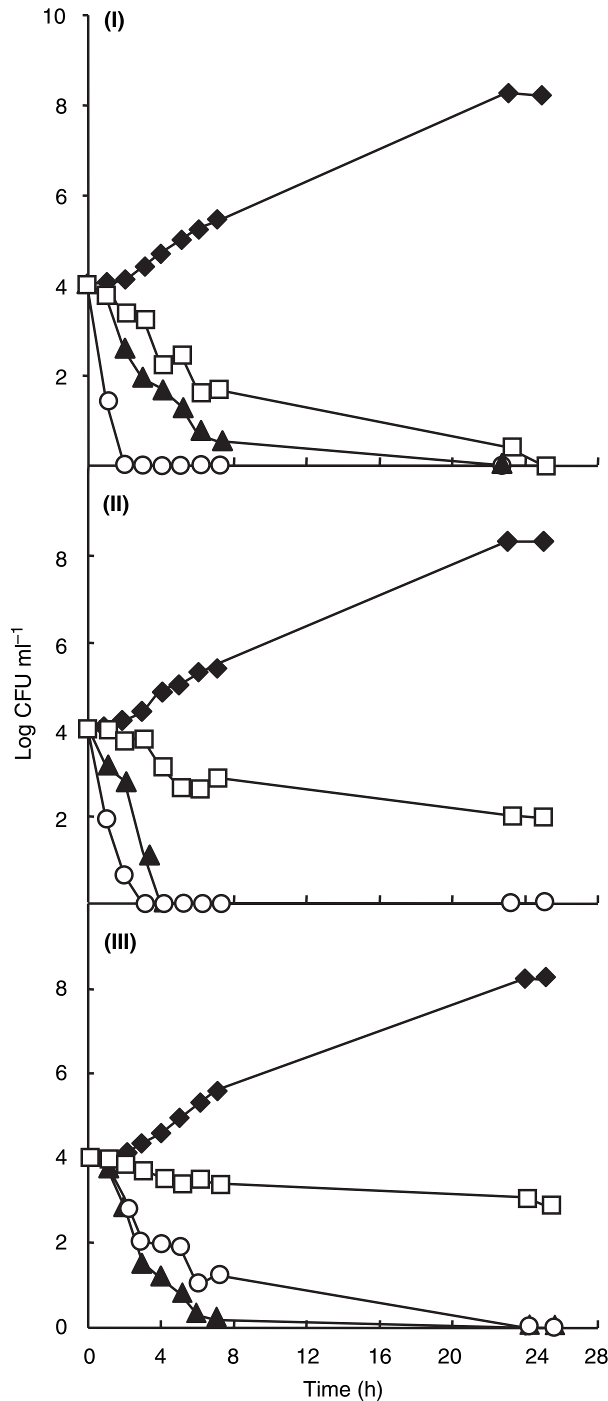

Figure 1 Fate of *Listeria monocytogenes* in aqueous extracts prepared from packaged iceberg lettuce shreds stored aerobically for 0 day (◆), 1 day (□), 2 days (○) and 3 days (▲) at 15°C respectively. Results of three experiments (I, II and III) are shown.

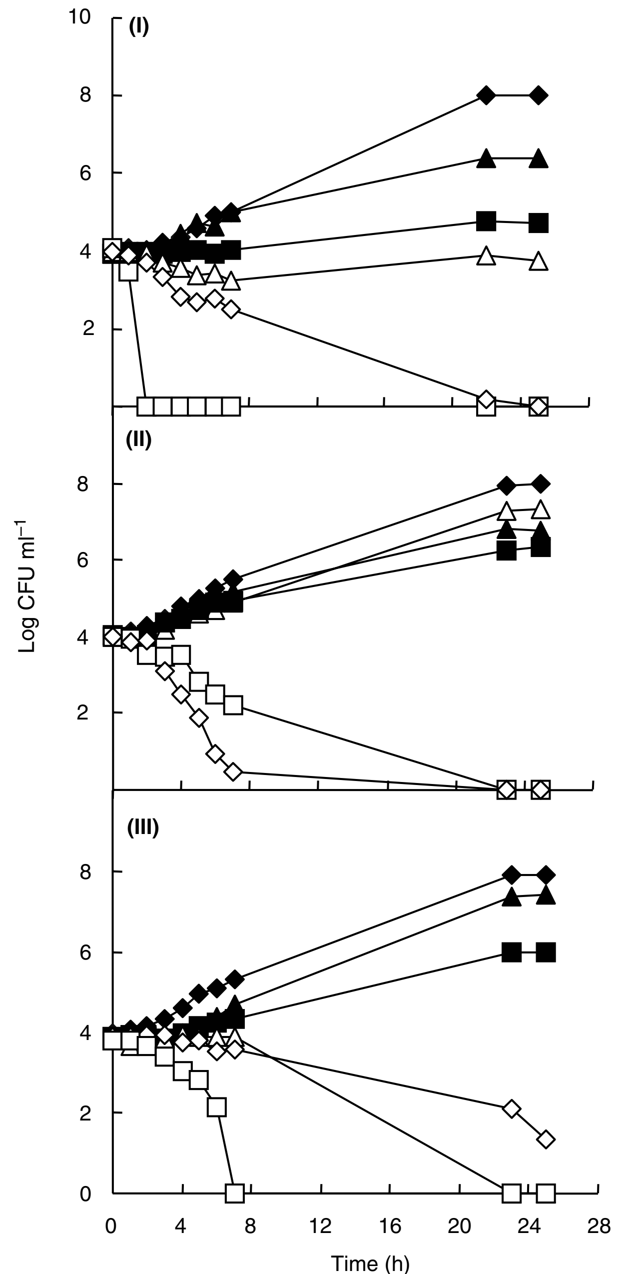

Figure 2 Fate of *Listeria monocytogenes* in aqueous extracts prepared from packaged iceberg lettuce shreds stored aerobically at 15°C after a 3-min wash in chlorinated (100 μg ml^{-1} NaOCl) water. Extracts prepared from unwashed lettuce stored at day 0 (◆) and after 3 days (◇); lettuce washed at 4°C at day 0 (■) and after 3 days storage (□) or at 47°C at day 0 (▲) and after 3 days (△). Results of three experiments (I, II and III) are shown.

unchanged in untreated lettuce shreds inoculated immediately after packaging. In contrast, a population decline was evident in shreds inoculated after 3 days in storage, an observation that hinted at possible attenuation by the background microflora as the latter accounted for a considerably larger portion of the total aerobic microbial population. However, results obtained with lettuce subjected to the wash treatments indicated that competition

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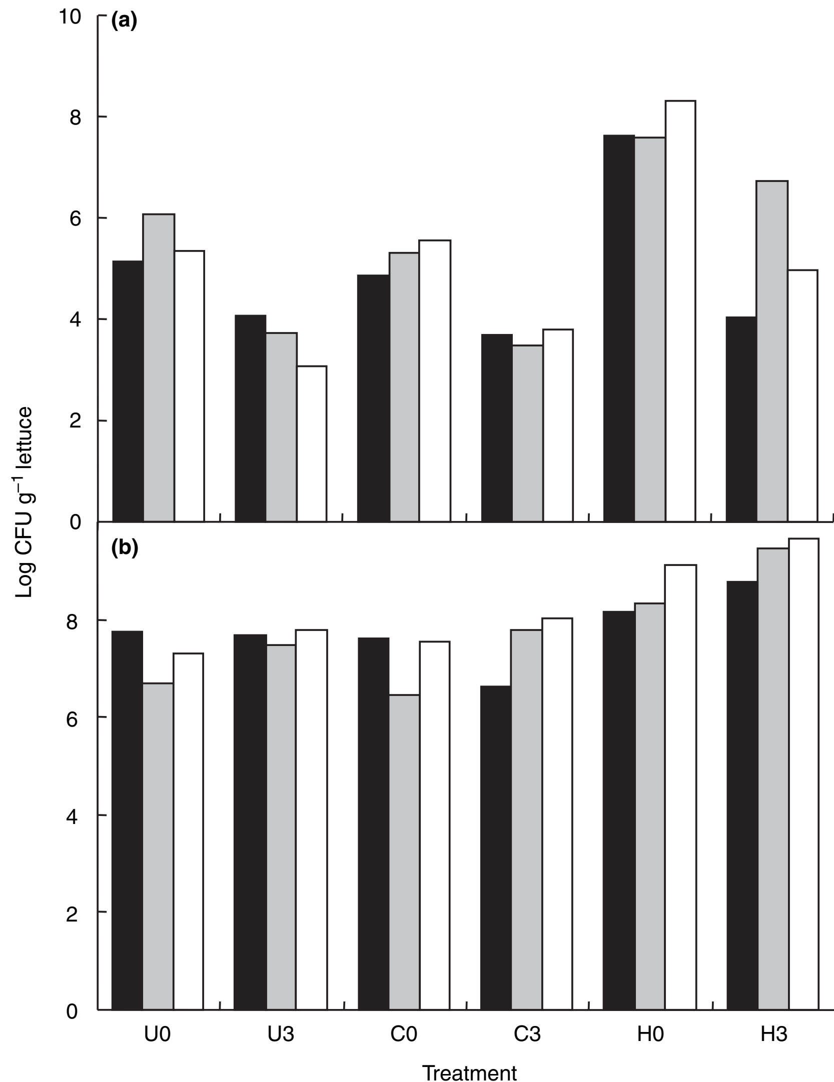

Figure 3 *Listeria monocytogenes* (a) and total aerobic microbial populations (b) in packaged lettuce shreds stored for 3 days at 15°C after inoculation. Treatments: U0 and U3, untreated lettuce inoculated after one and three days at 15°C; C0 and C3, inoculated after one and three days at 15°C following a 3-min wash at 4°C in chlorinated (100 μg ml^{-1} NaOCl) water; H0 and H3, inoculated after 1 and 3 days at 15°C following a 3-min wash at 47°C in chlorinated water. Results of three replicate experiments are shown.

alone could not account for the fate of the test strain. Washing in cold chlorinated water prior to inoculation had no effect on the fate of *L. monocytogenes* despite immediate reductions in background microbial populations of 1–2 log CFU g^{-1} (data not shown). Furthermore, the test strain grew rapidly in lettuce subjected to a heat shock despite background populations of log 8–9 CFU g^{-1}. Hence intrinsic physiological alterations mitigated by heat shocks prior to packaging clearly influenced the behaviour of *L. monocytogenes* in packaged cut lettuce. Mild heat shocks are known to decrease PAL activity and the synthesis of phenolic compounds that accumulate in wounded lettuce tissues, including cinnamic, chlorogenic, isochlorogenic, caffeic, caffeoyltartaric and dicaffeoyl tartaric acids (Ke and Saltveit 1988, 1989; Peiser *et al.* 1998; Fukumoto *et al.* 2002). Interestingly, several of these compounds exhibit antimicrobial activity *in vitro* (Barber *et al.*, 2000; Wen *et al.* 2003). The contribution of phenolic compounds to the inhibitory effect of cut lettuce tissue was verified by inoculation of the experimental strain into a phenolic-depleted aqueous extract. Results reported in Fig. 4 show that removal of 95% of phenolic compounds from lettuce extract (from 13·16 mg l^{-1} expressed as chlorogenic acid to 2·62 mg l^{-1} after separation) did not prevent inhibition of *L. monocytogenes*.

The inhibitory principle elicited in cut iceberg lettuce tissue under aerobic conditions remains unidentified and is the subject of continuing investigations in our laboratory. A phenolic nature has not been ruled out as some of the less hydrophobic chemical species in this complex group of phytochemicals may not be retained on a C18 column. In addition, several have well-defined functions in the defence of plants against microbial infection,

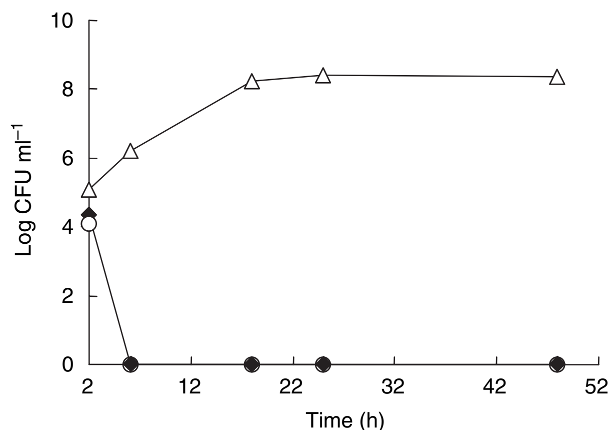

Figure 4 Fate of *Listeria monocytogenes* in aqueous extracts prepared from fresh lettuce shreds (△), after 3 days storage at 15°C before (◆) and after passage through a C18 column.

although comparatively little is known about their role in lettuce. One notable exception is the phytoalexin lettucenin A, a sesquiterpene lactone that is induced following microbial challenge by plant pathogens, particularly Gram-negative species such as *Pseudomonas cichorii* (Bestwick *et al.* 1995; Bennett *et al.* 2002). Whether other constitutive or induced phytoalexins exist in wounded iceberg lettuce tissues is unknown. The classical plant pathology literature describes many other classes of compounds active in plant defence mechanisms, including a range of phenolics, fatty acid derivatives, amino acid metabolites, peptides and proteins (Walker 1994). Clearly, their influence on the behaviour of human pathogens such as *L. monocytogenes* in plant foods remains to be defined. The evidence presented herein revealed the presence of a wound-associated factor with antilisterial activity that influenced the fate of the species in packaged cut lettuce. Furthermore, there were indications that treatments designed for improved quality retention may lessen the antilisterial activity. These observations point to the relevance of additional research aimed at characterization of the interaction between *L. monocytogenes* and plant foods that are distributed in a fresh-cut format. Moreover, a more complete understanding of the ecology of this psychotrophic pathogen in packaged, fresh horticultural products is desirable for the development of appropriate processing strategies to minimize the risk associated with growth during refrigerated storage.

References

Barber, M.S., McConnell, V.S. and DeCaux, B.S. (2000) Antimicrobial intermediates of the general phenylpropanoid and lignin specific pathways. *Phytochem* **54**, 53–56.

Bestwick, L., Bennett, M.H., Mansfield, J.W. and Rossiter, J.T. (1995) Accumulation of the phytoalexin lettucenin a and changes in 3-hydroxy-3-methylglutaryl coenzyme a reductase activity in lettuce seedlings with the red spot disorder. *Phytochemistry* **39**, 775–777.

Bennett, M.H., Mansfield, J.W., Lewis, M.J. and Beale, M.H. (2002) Cloning and expression of sesquiterpene synthase genes from lettuce (*Lactuca sativa* L.). *Phytochemistry* **60**, 255–261.

Beuchat, L.R. and Brackett, R.E. (1990) Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. *J Food Sci* **55**, 755–758,

Brecht, J.K. (1995) Physiology of lightly processed fruits and vegetables. *J Hort Sci* **30**, 18–22.

Camm, E.L. and Towers, G.H.N. (1973) Phenylalanine ammonia lyase. *Phytochemistry* **12**, 961–973.

Cantos, E., Espin, J.C. and Tomas-Barberan, F.A. (2001) Effect of wounding on phenolic enzymes in six minimally processed lettuce cultivars upon storage. *J Agric Food Chem* **49**, 322–330.

Delaquis, P.J., Stewart, S., Toivonen, P.M.A. and Moyls, A.L. (1999) Effect of warm, chlorinated water on the microbial flora of shredded iceberg lettuce. *Food Res Int* **32**, 7–14.

Delaquis, P.J., Stewart, S., Cazaux, S. and Toivonen, P. (2002) Survival and growth of *Listeria monocytogenes* and *Escherichia coli* O157 : H7 in ready-to-eat iceberg lettuce washed in warm, chlorinated water. *J Food Prot* **65**, 459–464.

Farber, J.M., Wang, S.L., Cai, Y. and Zhang, S. (1998) Changes in populations of *Listeria monocytogenes* inoculated on packaged fresh-cut vegetables. *J Food Prot* **61**, 192–195.

Francis, G.A. and O'Beirne, D. (1997) Effects of gas atmosphere, antimicrobial dip and temperature on the fate of *Listeria innocua* and *Listeria monocytogenes* on minimally processed lettuce. *Int J Food Sci Technol* **32**, 141–151.

Fukumoto, L.R., Delaquis, P.J. and Toivonen, P.M.A. (2002) Effect of water wash temperature and chlorination on phenolic metabolism and browning of stored iceberg lettuce photosynthetic and vascular tissues. *J Agric Food Chem* **50**, 4503–4511.

Jacxsens, L., Devlieghere, F., Falcato, P. and Debevre, J. (1999) Behavior of *Listeria monocytogenes* and *Aeromonas* spp. on fresh-cut produce packaged under equilibrium-modified atmosphere. *J Food Prot* **62**, 1128–1135.

Kang, H.M. and Saltveit, M.E. (2003) Wound-induced increases in phenolic content of fresh-cut lettuceis reduced by a short immersion in aqueous hypertonic solutions. *Postharvest Biol Technol* **29**, 271–277.

Ke, D. and Saltveit, M.E. (1988) Plant hormone interaction and phenolic metabolism in the regulation of russet spotting in iceberg lettuce. *Plant Physiol* **88**, 1136–1140.

Ke, D. and Saltveit, M.E. (1989) Wound-induced ethylene production, phenolic metabolism and susceptibility to russet spotting in iceberg lettuce. *Physiol Plant* **76**, 412–418.

Li, R., Brackett, R.E., Chen, J. and Beuchat, L.R. (2002) Mild heat treatment of lettuce enhances growth of *Listeria monocytogenes* during subsequent storage at 5°C or 15°C. *J Appl Microbiol* **92**, 269–275.

Loaiza-Velarde, J.G., Tomas-Barberan, F.A. and Saltveit, M.E. (1997) Effect of intensity and duration of heat-shock treatments on wound-induced phenolic metabolism in iceberg lettuce. *J Am Soc Hort Sci* **122**, 873–877.

Loaiza-Velarde, J.G. and Saltveit, M.E. (2001) Heat shocks applied either before or after wounding reduce browning of lettuce leaf tissue. *J Am Soc Hort Sci* **126**, 227–234.

López-Gálvez, G., Saltveit, M. and Cantwell, M. (1996) Wound-induced phenylalanine ammonia lyase activity: factors affecting its induction and correlation with the quality of minimally processed lettuces. *Postharvest Biol Technol* **9**, 223–233.

Peiser, G., López-Gálvez, G., Cantwell, M. and Saltveit, M.E. (1998) Phenylalanine ammonia lyase inhibitors control browning of cut lettuce. *Postharvest Biol Technol* **14**, 171–177.

Saltveit, M.E. (2000) Wound induced changes in phenolic metabolism and tissue browning are altered by heat shock. *Postharvest Biol Technol* **21**, 61–69.

Singleton, V.L. and Rossi, J.A. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* **16**, 144–158.

Steinbruegge, E.G., Maxcy, R.B. and Liewen, M.B. (1988) Fate of *Listeria monocytogenes* on ready to serve lettuce. *J Food Prot* **51**, 596–599.

Walker, J.R.L. (1994) Antimicrobial compounds in food plants. In *Natural Antimicrobial Systems and Food Preservation* ed. Dillon, V.M. and Board, R.G. pp. 181–204. Wallingford, UK: CAB International.

Wen, A., Delaquis, P.J. and Stanich, K. (2003) Antilisterial activity of selected phenolic acids. *Food Microbiol* **20**, 305–311.