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Minimal processing of iceberg lettuce has no substantial influence on the survival, attachment and internalization of *E. coli* O157 and *Salmonella*

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ABSTRACT

The influence of a selection of minimal processing techniques (sanitizing wash prior to packaging, modified atmosphere, storage conditions under light or in the dark) was investigated in relation to the survival of, attachment to and internalization of enteric pathogens in fresh produce. Cut Iceberg lettuce was chosen as a model for fresh produce, *Escherichia coli* O157:H7 (*E. coli* O157) and *Salmonella enterica* were chosen as pathogen models. Care was taken to simulate industrial post-harvest processing. A total of 50 ± 0.1 g of fresh-cut Iceberg lettuce was packed in bags under near ambient atmospheric air with approximately 21% O_2 (NAA) conditions or equilibrium modified atmosphere with 3% O_2 (EMAP). Two lettuce pieces inoculated with *E. coli* O157 BRMSID 188 or *Salmonella* Typhimurium labeled with green fluorescent protein (GFP) were added to each package. The bags with cut lettuce were stored under either dark or light conditions for 2 days at 7 °C. The pathogens' capacity to attach to the lettuce surface and cut edge was evaluated 2 days after inoculation using conventional plating technique and the internalization of the bacteria was investigated and quantified using confocal microscopy. The effect of a sanitizing wash step (40 mg/L NaClO or 40 mg/L peracetic acid + 1143 mg/L lactic acid) of the cut lettuce prior to packaging was evaluated as well. Our results indicate that both pathogens behaved similarly under the investigated conditions. Pathogen growth was not observed, nor was there any substantial influence of the investigated atmospheric conditions or light/dark storage conditions on their attachment/internalization. The pathogens attached to and internalized via cut edges and wounds, from which they were able to penetrate into the parenchyma. Internalization through the stomata into the parenchyma was not observed, although some bacteria were found in the substomatal cavity. Washing the cut edges with sanitizing agents to reduce enteric pathogen numbers was not more effective than a rinse with precooled tap water prior to packaging. Our results confirm that cut surfaces are the main risk for postharvest attachment and internalization of *E. coli* O157 and *Salmonella* during minimal processing and that storage and packaging conditions have no important effect.

1. Introduction

Concerns have been raised about the food safety of fresh produce, such as cantaloupe, herbs, lettuce, tomatoes, spinach and sprouts, due to the fact that these items are consumed raw. Numerous outbreaks with *Escherichia coli* O157:H7 (*E. coli* O157) and *Salmonella enterica* have been linked to consumption of fresh produce, consequently fresh produce are considered as high-risk food by the U.S. Food and Drug Administration (Klein et al., 2009). These enteric pathogens are able to attach to and even internalize in the products which makes them very difficult to remove (Erickson, 2012). In order to improve the convenience, minimal processed bagged fresh produce products are developed relying on modified atmosphere packaging (MAP, MA packaging) and sanitizing washes to maintain quality and ensure safety of fresh produce for consumers. However, the influence of minimal processing on pathogen behavior, in particular attachment and internalization still needs further study as it is not fully known to what extent these interventions can influence the risk.

By applying MAP, the respiration rate of lettuce is reduced due to the low O_2 percentage in the package which retards browning and inhibits

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the outgrowth of aerobic spoilage bacteria (Posada-Izquierdo et al., 2012). It was already shown that this may induce acid resistance of *E. coli* O157 on lettuce but only when stored at growth permissive temperatures (≥15 °C) (Chua et al., 2008). Furthermore, the virulence of *E. coli* O157:H7 on MA packed lettuce at this temperature was shown to be lower in comparison with near ambient air (NAA) conditions (Sharma et al., 2011). In addition, it was demonstrated that equilibrium MA packaging (EMAP) (10% O_2, 10% CO_2, and 80% N_2) had an antimicrobial effect on indigenous lettuce microbiota, but not on *Salmonella* and even favored the survival of this pathogen (Horev et al., 2012). Also, growth and survival of *E. coli* O157:H7 in manure and slurry were shown to be promoted under anaerobic conditions (Semenov et al., 2011). Thus far, only one study has investigated the combination of the effect of MAP (and temperature and respiration rate) on the attachment and internalization of *E. coli* O157 in fresh cut lettuce (Takeuchi et al., 2001). In the latter study, only the cut edges of lettuce were investigated in a lab-scale set-up. Takeuchi et al. (2001) did not report a relation between attachment or penetration and respiration rate of the lettuce and they only found small differences in attachment between the different atmospheric conditions and temperatures.

In the present study the influence of MA packaging on the survival, attachment and internalization of *Salmonella* and *E. coli* O157 on fresh cut Iceberg lettuce was investigated in order to identify conditions that present a lower risk to consumers such as low attachment degree of pathogens, low degree of pathogen internalization or higher susceptibility of the pathogens for sanitizers. Special attention was paid to simulate commercial MA packaging (equilibrium MAP, 3% O_2, 97% N_2, 7 °C). As it was already shown that the attachment of the pathogen may be different for the cut edge or the surface of a cut leaf piece (Kroupitski et al., 2009b; Seo and Frank, 1999; Takeuchi and Frank, 2001; Takeuchi et al., 2000, 2001), both leaf regions were investigated. Since Kroupitski et al. (2009a) revealed that *Salmonella* is attracted to nutrients produced by photosynthetically active cells of cut lettuce, and bagged fresh-cut lettuces are exposed to light in food stores, storage conditions (light or dark) were taken into account as well, In addition, this effect was for the first time investigated on *E. coli* O157 and cut lettuce. The classical plate count technique as well as quantitative confocal laser scanning microscopy were used in the present study to evaluate and quantify the effect of high (necessary for monitoring with confocal laser microscopy) or moderate pathogen levels. In addition, the effect of the combination of a sanitizing wash step (40 mg/L NaClO or 40 mg/L peracetic acid + 1143 mg/L lactic acid) of the cut lettuce prior to MA-packaging was studied on a selection of conditions that were identified in this study to impose a high risk of pathogen internalization.

2. Materials and methods

2.1. Influence of MAP, light, cut edge/surface, and inoculum density

2.1.1. Bacterial strains and growth conditions

Chromosomally green fluorescent protein (GFP) labeled *Salmonella enterica* (serovar Typhimurium) and *E. coli* O157 strain BRMSID 188 GFP were used. The wildtype *Salmonella* Typhimurium was isolated from freshwater sediments and chromosomally labeled with a stable variant of GFP by means of the pUT mini-Tn5 Km transposon by tri-parental mating. The strain was kindly donated by Prof. Venter (Division of Natural Resources and the Environment, CSIR, Pretoria 0001, South Africa) and described in (Burke et al., 2008). *E. coli* O157:H7 BRMSID188 GFP was kindly donated by Dr. Susan Bach (Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Canada) and described in (Dinu and Bach, 2011). Briefly, the wild type was isolated from bovine and chromosomally labeled with a stable variant of GFP by means of Tn7-transposon. Both strains are resistant against 100 μg/mL kanamycin.

Reference stocks were stored at −75 °C in tryptone soy broth (TSB; Oxoid, Basingstoke, England), supplemented with 50 μg/mL kanamycin (TSB-K) and 15% glycerol (Prolabo, Heverlee, Belgium). Stock cultures were kept at 4 °C on tryptone soy agar (TSA, Oxoid, Basingstoke, England) slants supplemented with 50 μg/mL kanamycin (TSA-K). Working cultures were prepared by loop inoculation in 10 mL of TSB-K and statically incubated for 6 ± 1 h at 37 °C. Then, 2 mL of this inoculum was transferred to 200 mL TSB-K in a 500 mL bottle and incubated at 37 °C for 18 ± 1 h, using an orbital shaker (200 rpm, Yellowline RS/OS 10 Control, IKA-Werke GmbH&Co, Staufen, Germany).

The optical density (650 nm) of the bacterial cultures was measured in triplicate in a 96-well plate using a spectrophotometer (Versamax, Molecular Devices, Wokingham, UK). Bacterial suspensions (~log 9.5 CFU/mL or ~log 5.5 CFU/mL) were washed twice with sterile distilled water by centrifugation (2900 ×*g* for 10 min at room temperature) (Sigma 4K15, SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany). Cells in the pellet were resuspended in 100 mL sterile distilled water and the bacterial suspension was stored at 7 °C until use (max 1 h). The cell population of the inoculum was determined by plating 10-fold serial dilutions in physiological peptone salt solution (PPS) on TSA-K (18–24 h, 37 °C).

2.1.2. Inoculation of lettuce pieces

Iceberg lettuce was obtained from a local vegetable supplier (Ghent, Belgium). The lettuce heads were weighed, cut in two and the maturity stage was determined based on the lettuce head density chart. The three (for younger maturity stage) or four to five (for higher maturity stage) outer leaves of the lettuce were removed. The next three leaves were used to cut leaf pieces of approximately 4.5 cm × 4.5 cm on the top-region of the leaf (Fig. 1). A triangular mark was cut out in the lower left corner of the lettuce piece, when the lettuce piece was lying with the abaxial side on the cutting board and with the top ruffle of the leaf facing away from the operator. Subsequently, the leaf pieces were inoculated by submerging them individually for 2 s in the bacterial suspension using a sterile forceps. Afterwards, the leaf pieces were drained on a paper towel to remove the excess of bacterial suspension and stored at 7 °C until packed (max 1 h).

2.1.3. Minimal processing of uninoculated lettuce

Iceberg lettuce was processed as described by Lopez-Galvez et al. (2013) with some modifications. Briefly, after its reception in the lab, lettuce was kept at 4 °C for max. 2 h before processing. Outer leaves and core were removed and lettuce was cut in pieces of approximately 4.5 cm × 4.5 cm by means of stainless steel knives in clean conditions. About 1 kg of untreated fresh-cut lettuce was washed in 10 L cold tap water (7 °C) for 1 min. Subsequently, the product was rinsed again in 10 L tap water at 7 °C for 1 min. Finally, the cut lettuce was drained by centrifuging for 1 min using a manually operated centrifuge (16 L). All the processing steps were performed in a cold room at 7 °C.

2.1.4. Packaging and storage

In the current study a fill weight of 50 ± 0.1 g was used. Therefore, the bags (19 cm × 19 cm) were first filled with ±25 g of uninoculated minimally processed lettuce. Subsequently, two inoculated leaf pieces (1.7 ± 0.7 g) were added to the bag with a sterile forceps and the bag was further filled with uninoculated minimally processed lettuce until a weight of 50 ± 0.1 g was reached. Equilibrated modified atmosphere packaging (EMAP, 3% O_2, 97% N_2) conditions were performed with a packaging foil (60 LB PLAIN, Amcor-Flexibles, Ledbury, Herefordshire, UK) with an O_2 permeability of 1000 mL $O_2/m^2 \cdot d \cdot atm$ measured at ASTM D1434, 23 °C, 0% RH. The foil consisted of a 30 μm printable clear polypropylene layer and a 30 μm clear polyethylene layer. Then, the packages were flushed with 2.5–3% O_2 (Freshline, Air Products, Brussels, Belgium) and 97–97.5% N_2 (RT-X50S-Food, Air Products, Brussels, Belgium) as initial gas atmosphere by means of a gas packaging unit consisting of a gas mixer (WITT KM 100-4 MEM, Witt-Gasetechnic, Witten, Germany). The near ambient air atmospheric (NAA) conditions were realized with a high barrier packaging foil (NX90, Euralpack,