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Survival of *Listeria monocytogenes* on fresh and frozen strawberries

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Abstract

Cut or intact surfaces of fresh strawberries were spot inoculated with a five-strain cocktail of nalidixic-acid resistant *Listeria monocytogenes* (10^6 (low inoculum) and 10^8 (high inoculum) CFU per three-berry sample). Inoculated strawberries were dried for 1 h at 24 °C and were stored in loosely closed containers at 4 or 24 °C. An initial population reduction of approximately 0.6 and 1.2 log cycles, high and low inoculum, respectively, was observed on intact but not cut berries after the 1-h drying period. A decrease of 1.4 and 3.3 log cycles per intact sample was observed over 48 h for the high and low inoculum, respectively, when stored at 24 °C. When held at 4 °C, a reduction of approximately 3 log cycles per intact-berry sample was observed for both inocula over the 7-day storage period. Populations on cut surfaces remained constant at both temperatures and both inoculum densities throughout the storage period. Sliced, inoculated strawberries (6.7 log CFU/25-g sample) with or without 20% sucrose were frozen at −20±2 °C. After 28 days of frozen storage, populations of *L. monocytogenes* determined on tryptose phosphate agar supplemented with nalidixic acid (TPAN) had declined by 0 to 1.2 log cycles, with and without 20% sucrose, respectively. Counts on modified Oxford agar supplemented with nalidixic acid were significantly ($P \leq 0.05$) lower (0.5 to 1.8 log CFU/g) than on TPAN indicating that some cell injury had occurred. Results of this study indicate that *L. monocytogenes* is capable of survival but not growth on the surface of fresh intact or cut strawberries throughout the expected shelf life of the fresh fruit and can survive on frozen strawberries for periods of at least 4 weeks. On whole strawberries held at 24 °C, significantly faster declines ($P \leq 0.05$) of *L. monocytogenes* were observed when lower rather than higher inoculum levels were applied.

Keywords: Strawberry; *Listeria monocytogenes*; Fruit; Frozen; Pathogen

1. Introduction

For maximum shelf life, strawberries are usually harvested directly into retail containers and then rapidly cooled before distribution (Mitcham and Mitchell, 2002). They are not washed because even small amounts of moisture can result in rapid decay of

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the fruit primarily due to the growth of *Botrytis cinerea* (Mitcham and Mitchell, 2002). In contrast, berries picked for frozen processing are usually hulled in the field, transported to the processing facility, and washed prior to freezing. Berries may be commercially frozen with the addition of up to 30% sucrose or other sugars (Boyle and Wolford, 1968; Perkins-Veazie and Collins, 1995). Home freezing instructions for strawberries also include the addition of 0% to 23% sucrose added in granular form or as a prepared syrup (Andress and Harrison, 1999). In addition to direct consumption, both fresh and frozen strawberries are often used in other products that receive no thermal treatment after their addition.

The risks associated with consumption of fresh or frozen strawberries are generally considered to be low. Reported foodborne illnesses associated with strawberries have been limited to three outbreaks of hepatitis A in commercially frozen berries (Dougherty et al., 1965; Niu et al., 1992; Hutin et al., 1999). Outbreaks associated with bacterial pathogens have not been reported with the exception of an outbreak of listeriosis attributed to a mixture of blueberries, nectarines, and strawberries (see Schlech, 1996) and one attributed to *Staphylococcus aureus* (see Sivapalasingam et al., 2004). However, details of the investigation including possible sources of contamination were not provided.

Listeria monocytogenes is commonly associated with plant matter and soil and it has been suggested that it may be more common in those fruits and vegetables grown in close association with the soil (Brackett, 1999). Although *L. monocytogenes* has been isolated from a variety of raw vegetables (see Harris et al., 2003) its association with fruits or acidic vegetables is less well documented. Using standard enrichment procedures, Heisick et al. (1989) was unable to isolate *Listeria* spp. in 92 samples of tomatoes (25 g each) collected from retail markets. Parish and Higgins (1989b) failed to isolate *Listeria* spp. in 25-g samples of 100 retail samples of reconstituted single-strength orange juice collected from across the United States and Canada. However, *L. monocytogenes* was detected by enrichment in two (25 g each) samples of unpasteurized apple (pH 3.78) and apple/raspberry juice (pH 3.75) out of 50 retail juice samples tested (Sado et al., 1998). More recently, *L. monocytogenes* serogroup 4 was isolated by enrichment from 1 of 173 strawberries obtained from Norwegian retail markets (Johannessen et al., 2002).

Listeriosis is more commonly associated with food products that support the growth of *L. monocytogenes* to levels in excess of 100 CFU/g (Chen et al., 2003). Growth of *L. monocytogenes* has been demonstrated in a number of vegetables under refrigerated and ambient conditions (see Harris et al., 2003) and in non-acidic fruits (Ukuku and Fett, 2002). Growth has also been demonstrated on the outer surface of acidic fruits such as tomatoes (Beuchat and Brackett, 1991) and peeled Hamlin oranges (Pao et al., 1998) when stored at greater than 20 °C.

There are no previous reports on the growth or survival of *L. monocytogenes* inoculated onto strawberries. The objective of the current study was to evaluate the fate of *L. monocytogenes* on cut and whole strawberries stored at −20, 4, and 24 °C.

2. Materials and methods

2.1. Strawberries

Fresh, ripe, unblemished strawberries were purchased at a local supermarket either the day before or the day of an experiment. The strawberries were stored at 4 °C until approximately 1 h before the experiment when they were transferred to ambient temperature (24±2 °C).

The strawberries used in this study were left whole, or cut in slices. Detailed methods were reported previously (Knudsen et al., 2001). Briefly, whole strawberries were used unhulled and unwashed. For some experiments, strawberries were hulled by aseptically removing the leafy part of the fruit (calyx). The hulled strawberries were cut on one side of the berry to expose internal flesh or were cut into approximately 0.6-cm slices. Sliced strawberries (25±2 g) were placed into 15×23 cm filter bags (Nasco, Inc., Modesto, CA). Sliced strawberries were frozen with or without the addition of 20% sucrose (wt/wt).

2.2. Test strains and media preparation

Dr. Larry Beuchat, University of Georgia, provided the bacterial strains used in this study. A cocktail was prepared with the following five *L. monocytogenes*

isolates: V7 (serotype 4b, milk), LCDC 81-861 (serotype 4b, raw cabbage), Scott A (serotype 4b, human isolate associated with milk), 101 M (unknown serotype, beef), and 108 M (unknown serotype, beef). All strains had been adapted to grow in the presence of 50 μg/ml nalidixic acid. Viable populations of *L. monocytogenes* were recovered on tryptose phosphate agar (Difco, Detroit, MI) supplemented with sodium pyruvate (1 g/l) (Fisher, Fair Lawn, NJ) and nalidixic acid (50 μg/ml) (Sigma, St. Louis, MO) (TPAPN) and Modified Oxford Agar (Difco) supplemented with nalidixic acid (50 μg/ml) (MOXN). Nalidixic acid was used to suppress the growth of the background populations found on uninoculated strawberries (Knudsen et al., 2001).

2.3. Inoculum preparation

Prior to each experiment, frozen stock cultures were grown at 37 °C for 24 h on tryptose phosphate agar supplemented with nalidixic acid (50 μg/ml) (TPAN) and an isolated colony was cultured in tryptose phosphate broth supplemented nalidixic acid (50 μg/ml) (TPBN). After incubation at 37 °C for two consecutive 18 to 24-h intervals, single loop transfers were made in TPBN. Overnight (18 h) cultures were collected by centrifugation and suspended in 5% horse serum (Gibco, BRL, Grand Island, NY) to simulate the presence of organic material (Beuchat et al., 2001). The cocktail was prepared by combining equal portions of each strain to produce an inoculum of approximately 10^9 CFU/ml. When appropriate, the cocktail was further diluted 100 to 10,000-fold in 5% horse serum. Samples of the cocktail were plated onto MOXN and TPAPN to verify initial inoculum levels.

2.4. Inoculation procedure

Each strawberry was inoculated with 15 μl (2 to 4 drops) of the prepared *L. monocytogenes* cocktail on the intact side or cut surface. Strawberries were then held at room temperature for 1 h under a biological hood with the fan running to facilitate drying. Strawberries were either immediately analyzed or transferred to loosely closed plastic containers and incubated at 4±2 or 24±2 °C for 22 h, 1, 4, and 7 days or 2, 24, and 48 h, respectively.

Prepared bags of sliced strawberries (25 g) were inoculated with 15 μl of the *L. monocytogenes* cocktail and gently massaged for 60 s to ensure even dispersion of the bacteria. Samples were placed flat in a single layer in a freezer (−20±2 °C). Frozen samples were thawed by immersing the bag in a 25±2 °C water bath for 8±2 min.

2.5. Recovery of L. monocytogenes

Strawberries were macerated with 0.1 M phosphate buffer (0.1 M H_2KO_4P, 0.04 M $CaCO_3$, BBL, Becton Dickinson, Cockeysville, MD) by pummeling in a stomacher (Stomacher 400, Seward, England) for 90 s at low speed in filter stomacher bags. Subsequent dilutions were made using 0.1% peptone and the samples were plated using an Autoplater 4000 Spiral Plater (Spiral Biotech, Inc, Bethesda, MD). Presumptive *L. monocytogenes* were counted after incubation (37 °C for 48 h). *L. monocytogenes* was confirmed for some colonies by the presence of the enzymes hemolysin and catalase as described in the FDA Bacteriological Analytical Manual (Hitchins, 1998).

2.6. Statistics

Triplicate samples were analyzed at each time point and all experiments were replicated twice. Data were analyzed using statistical analysis software (Statistical Analysis Systems Institute, ver. 6.0, Cary, NC). Statistically significant differences ($P \leq 0.05$) between mean values were determined using analysis of variance.

3. Results

3.1. Survival of L. monocytogenes on whole and cut strawberries stored at 24 °C

Initial inoculation levels of approximately 7.5 log CFU (high) and 5.6 log CFU (low) per sample, respectively were achieved on both whole and cut strawberries (Fig. 1a and b). The inoculum was absorbed into the strawberry within approximately 20 min after inoculation. Based on previous studies with *Salmonella* and *Escherichia coli* O157:H7 (Knudsen et al., 2001), a 1-h drying time was used for all experiments.

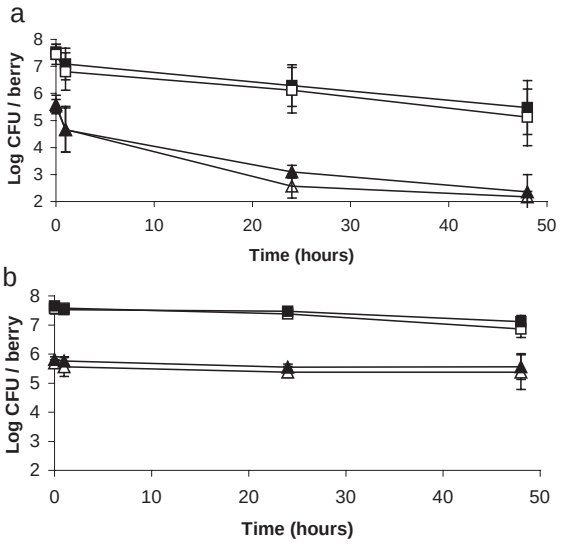

Fig. 1. (a) Survival of *L. monocytogenes* on whole strawberries inoculated with approximately 10^8 (square symbols) or 10^6 (triangular symbols) CFU/berry stored at 24 °C and plated onto TPAPN (closed symbols) and MOXN (open symbols). Values are the average of triplicate samples from each of two experiments (n=6). Error bars represent the standard deviation. (b) Survival of *L. monocytogenes* on cut strawberries inoculated with approximately 10^8 (square symbols) or 10^6 (triangular symbols) CFU/berry stored at 24 °C and plated onto TPAPN (closed symbols) and MOXN (open symbols). Values are the average of triplicate samples from each of two experiments (n=6). Error bars represent the standard deviation.

When inoculated at high levels, populations on whole berries were reduced by 0.4 log CFU/sample to 7.1 log CFU/sample during the 1-h drying period (Fig. 1a). A further significant decline ($P \leq 0.05$) of 1.0 log CFU/sample was observed over the 48-h storage period. Significantly larger initial and overall reductions were observed when berries were inoculated at the lower inoculum level. Populations decreased by approximately 1.0 log in the first hour and by an additional significant ($P \leq 0.05$) 2.2 log cycles after 48 h. Counts on TPAPN and MOXN were not significantly different ($P > 0.05$) with the exception of the 24-h samples, low-level inoculation ($P \leq 0.05$). Whole or cut strawberries were deemed inedible (soft and moldy) after the first 48 h when stored at 24 °C and the study was not extended beyond this time.

During the initial 1-h drying period, populations of *L. monocytogenes* did not decrease significantly ($P > 0.05$) on cut berries (Fig. 1b). A small (0.5 log CFU/sample) but significant decline ($P \leq 0.05$) was observed for the high inoculum level after 48 h of storage. Significant declines ($P > 0.05$) were not observed over the 48-h storage period at the low inoculum level (Fig. 1b). No significant difference ($P > 0.05$) was observed between counts on TPAPN and MOXN at any time point.

3.2. Survival of *L. monocytogenes* on whole and cut strawberries stored at 4 °C

Whole and cut strawberries were inoculated with high (approximately 7.7 log CFU/sample) or low (approximately 5.9 (whole) and 5.2 (cut) log CFU/sample) levels of the *L. monocytogenes* cocktail and stored for up to 7 days at 4 °C (Fig. 2a and b). Within

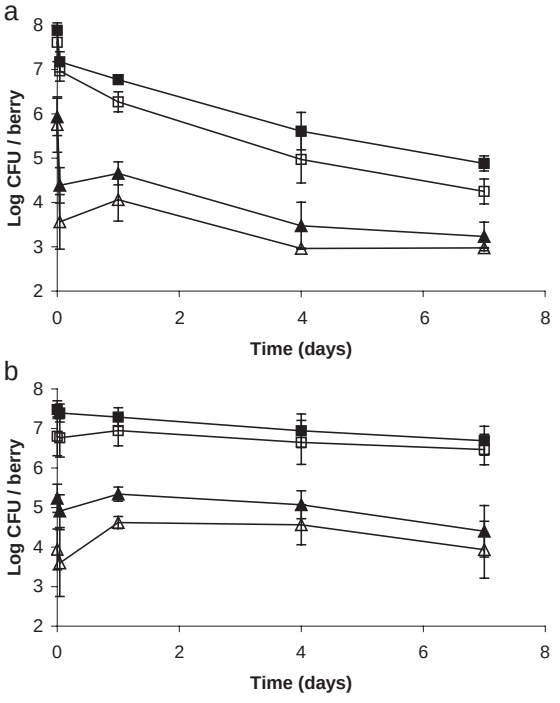

Fig. 2. (a) Survival of *L. monocytogenes* on whole strawberries inoculated with approximately 10^8 (square symbols) or 10^6 (triangular symbols) CFU/berry stored at 4 °C and plated onto TPAPN (closed symbols) and MOXN (open symbols). Values are the average of triplicate samples from each of two experiments (n=6). Error bars represent the standard deviation. (b) Survival of *L. monocytogenes* on cut strawberries inoculated with approximately 10^8 (square symbols) or 10^6 (triangular symbols) CFU/berry stored at 4 °C and plated onto TPAPN (closed symbols) and MOXN (open symbols). Values are the average of triplicate samples from each of two experiments (n=6). Error bars represent the standard deviation.

the first hour, populations on intact berries decreased by 0.7 and 1.5 log CFU/berry for the high and low inoculum, respectively. At the high inoculum level, a significant ($P \leq 0.05$) total reduction (3 log CFU/sample) was observed after 7 days of storage (Fig. 2a). At the low inoculum level, a similar significant ($P \leq 0.05$) total reduction of 2.7 log CFU/berry was observed (Fig. 2a). Counts on MOXN were slightly (0.5 log CFU/berry) but significantly lower ($P \leq 0.05$) than counts on TPAPN at most time points indicating a consistent level of sublethal injury.

The *L. monocytogenes* cocktail survived well on the surface of cut strawberry samples, with a significant ($P \leq 0.05$) but less than a 1 log decline of viable organisms after 7 days of storage for both inoculum levels (Fig. 2b). Counts on MOXN were not significantly different ($P > 0.05$) from those on TPAPN at most time points. Whole or cut strawberries were deemed inedible (soft and moldy) after 7 days of storage at 4 °C and the study was not extended beyond this time.

3.3. Survival of *L. monocytogenes* on cut strawberries stored at −20 °C

A freezer temperature of −20 °C was selected for this study because home and retail freezers are typically maintained at or below −18 °C (Andress and Harrison, 1999). Sliced strawberries were inoculated with approximately log 6.7 CFU/25-g sample of the *L. monocytogenes* cocktail. Freezing had the greatest impact on *L. monocytogenes* within the first 24 h of storage when populations in strawberry samples without added sucrose decreased by almost 1 log cycle on TPAPN (Table 1). Populations of *L. monocytogenes* remained stable in strawberries with added sucrose but underwent a significant ($P \leq 0.05$) 1.2 log CFU/sample decline upon freezing in strawberries without sucrose over the 28-day storage period. Throughout the trial, and in all cases, significantly greater ($P \leq 0.05$) numbers of *L. monocytogenes* were recovered on TPAPN than on MOXN, indicating the presence of injured cells (Table 1).

Table 1
Survival of *L. monocytogenes* on strawberries frozen with or without 20% sucrose plated onto TPAPN and MOXN

Day	0% sucrose			20% sucrose		
	TPAPN	MOXN	Δ[a]	TPAPN	MOXN	Δ
0	6.7±0.2 A[b]	6.2±0.2 A	0.5	6.4±0.3 AB	5.6±0.6 AB	0.8
1	6.0±0.2 B	4.6±0.6 BD	1.8	6.5±0.1 AB	5.7±0.3 AB	0.8
7	5.8±0.3 BD	5.0±0.3 B	0.8	6.6±0.2 A	5.8±0.2 B	0.8
14	5.1±0.7 C	4.0±0.7 CE	1.1	6.5±0.2 AB	5.4±0.3 AC	1.1
21	5.5±0.4 CD	4.3±0.6 DE	1.2	6.4±0.4 B	5.4±0.4 AD	1.0
28	5.5±0.7 D	4.5±0.9 BD	1.0	6.4±0.3 AB	5.3±0.4 CD	1.1

Values are the average log CFU/g±standard deviation of triplicate samples from each of two experiments ($n=6$).

[a] The sub-lethally injured population of *L. monocytogenes* represented as the difference between recovery on TPAPN and MOXN.

[b] Mean values in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$).

4. Discussion

Listeriosis is more commonly associated with food products that support the multiplication of *L. monocytogenes* (Chen et al., 2003). Growth of *L. monocytogenes* has been demonstrated at refrigerated and ambient temperature in a variety of stored vegetable products (Berrang et al., 1989; see Harris et al., 2003). In fruits, demonstration of growth has been limited to intact surfaces and to ambient storage temperatures. For example, *L. monocytogenes* was capable of growing within 24 h on the surface of peeled Hamlin oranges stored at 24 °C (Pao et al., 1998) and within 48 h on the surface of intact whole tomatoes stored at 21 °C (Beuchat and Brackett, 1991). In contrast, in the current study, reductions of 2.4 to 3.2 log CFU/berry of *L. monocytogenes* occurred within 48 h on uncut strawberries stored at 24 °C. Different surface structures and access to adequate moisture or nutrients may explain the inability of *L. monocytogenes* to grow on uncut strawberries when compared to other fruit surfaces.

Survival of pathogens on produce surfaces is generally improved when storage temperatures are reduced to 10 °C or below. Reductions of *L. monocytogenes* on intact strawberries observed within 48 h at 24 °C were not achieved until 4 to 7 days of storage at 4 °C. Survival of *L. monocytogenes* was similarly enhanced on peeled Hamlin oranges stored at 4 and 8 °C (Pao et al., 1998) and on the surface of whole tomatoes stored at 10 °C (Beuchat and Brackett, 1991). To maximize shelf life, strawberries are rapidly cooled to 2 °C after harvest and are ideally shipped at 1 °C

(Mitcham and Mitchell, 2002). Survival of *L. monocytogenes* was not evaluated at these temperatures but is likely equal to or better than that observed at 4 °C. Levels of viable *L. monocytogenes* on refrigerated, intact strawberries at the point of consumption will depend upon initial contamination level, storage temperature, and length of storage.

In addition to storage temperature and time, growth and survival of *L. monocytogenes* is influenced by pH and type of acid (Sorrells et al., 1989). Strawberries used in the current study had a pH of approximately 3.6 to 3.8 (data not shown), well below the minimum of pH 4.4 to 4.5 determined for growth of *L. monocytogenes* in laboratory medium (Parish and Higgins, 1989a; Sorrells et al., 1989). Reductions of *L. monocytogenes* of up to 0.5 log CFU/berry were observed on cut strawberries stored for 48 h or 7 days at 24 or 4 °C, respectively. It is unlikely that populations of *L. monocytogenes* would change over the normal shelf life of cut strawberries sold at retail or prepared in the home or food service.

Survival of *L. monocytogenes* in orange serum was similar to that on cut strawberries. In orange serum adjusted to pH 3.6 or 3.8, populations were relatively stable over 7 days of storage at 4 °C and declined by 0.5 to 1 log CFU/ml within 48 h of storage at 30 °C. Rapid, approximately 4 to 5 log declines were observed in orange serum from 2 to 5 days of storage at 30 °C (Parish and Higgins, 1989a). However, these storage times far exceed the shelf life observed in the current study for strawberries stored at 24 °C. In contrast, rapid declines of *L. monocytogenes* were observed in apple cider (pH 3.3–3.5) stored at 4 °C (Roering et al., 1999). The number of viable *L. monocytogenes* declined from 10^5 or 10^6 CFU/ml to below 10 CFU/ml within 48 h. The lower pH or concentration of acid in the apple cider evaluated by Roering et al. (1999) might explain the more rapid reduction of *L. monocytogenes* populations in this system. The exposure of the organism to a juice system rather than a cut fruit surface may also explain differences observed in survival.

The survival of *L. monocytogenes* on intact and cut strawberries was similar to that previously reported for *E. coli* O157:H7 and *Salmonella* where a single inoculum level of 10^7 CFU/berry (Knudsen et al., 2001) or 10^4 CFU/g (Yu et al., 2001) was used. Like *L. monocytogenes*, populations of *Shigella* (Flessa and Harris, 2002) declined more rapidly during drying and storage when inoculated at lower (5.0 log CFU/berry) rather than higher (7.0 log CFU/berry) levels. Most laboratory studies evaluate the fate of relatively large concentrations of inoculated pathogens which may or may not reflect natural contamination loads. The potential for reduced survival at lower inoculum levels should be considered in microbial risk assessments. Researchers may also want to consider evaluating the impact of microbial load when determining survival of pathogens in food systems.

The survival of *L. monocytogenes* in frozen strawberries was similar to that previously reported for *E. coli* O157:H7. The survival of *E. coli* O157:H7 in sliced strawberries depended on the strain tested, the length of storage, and the presence or absence of sucrose. The short-term (30-day) survival of both pathogens was generally good in frozen strawberries, especially in the presence of 20% sucrose (Knudsen et al., 2001).

The complex and delicate nature of the strawberry surface impedes the removal of microorganisms after contamination. To maintain shelf life, washing of fresh strawberries is generally delayed to the point of consumption. However, washing steps are employed when commercially preparing strawberries for freezing. When washing strawberries, 1 to 2 log reductions are typically observed depending on the sanitizer used and type of inoculated pathogen (*Salmonella*, *E. coli* O157:H7, *L. monocytogenes*) (Schlitt-Dittrich et al., 2004; Lukasik et al., 2003; Yu et al., 2001). Reductions on cut strawberries are more difficult to achieve (Harris, unpublished). In one recently published study, 5 to 6 log reductions of inoculated *E. coli* O157:H7 and *L. monocytogenes* were reported for strawberries upon exposure to four different sanitizers (Rodgers et al., 2004). However, this magnitude of reduction has rarely been observed with produce (Beuchat and Ryu, 1997; Parish et al., 2003).

Strawberries should be considered a low risk for listeriosis because of their inability to support the growth of *L. monocytogenes*. Fresh or frozen strawberries are often added without further processing as an ingredient or to flavor other foods. This may be a concern if the food can support the growth of *L. monocytogenes*. Risk-reduction strategies for strawberries should focus on minimizing the potential for contamination along the food chain, a strategy

supported in a recent risk evaluation for this fruit (Nottermans et al., 2004).

Acknowledgements

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