

EFFECT OF RELATIVE HUMIDITY AND STORAGE TEMPERATURE ON THE BEHAVIOR OF *LISTERIA MONOCYTOGENES* ON FRESH VEGETABLES

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Received for Publication July 25, 2013
Accepted for Publication October 4, 2013

doi: 10.1111/jfs.12087

ABSTRACT

The survival and growth of *Listeria monocytogenes* on fresh lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*) epidermis and parsley (*Petroselinum sativum*) under different relative humidity (RH) conditions and storage temperatures was studied. Inoculated vegetable pieces were stored at 10C for 7 days, 20C for 5 days, and 30C for 24 h, and at 53 and 90% RH. *L. monocytogenes* was enumerated periodically by plate count. *L. monocytogenes* did not grow on lettuce or parsley, but either declined in number or remained approximately constant. At cooler temperatures, a negative effect of low humidity on survival was observed. *L. monocytogenes* grew on cucumber epidermis at all three temperatures, increasing from around 4.0 to around 5.5 log cfu/cm² when stored at 90% RH. Low RH negatively affected growth, limiting the increase to around 0.5 log cfu/cm² in most cases.

PRACTICAL APPLICATIONS

Fresh vegetables are increasingly important vehicles of foodborne pathogens. The majority of published studies on growth and survival of *Listeria monocytogenes* on vegetables have been conducted on lettuce leaves and few data are available on the influence of relative humidity (RH). Growth of *L. monocytogenes* may increase the risk to the consumer of some types of fresh vegetables. Maintaining lower RH during storage can decrease this risk, but may conflict with other product quality requirements. *L. monocytogenes* does not grow on undamaged leaf surfaces under the conditions tested: when reviewing the literature it is important to differentiate between whole and shredded/cut leaves.

INTRODUCTION

During recent decades, the consumption of ready-to-eat and minimally processed fruits and vegetables has increased. Between 1976 and 2009, per capita consumption of fresh vegetables in the United States increased by 52.7% (Cook 2011). Although listeriosis (the infection caused by *Listeria monocytogenes*) has been primarily associated with consumption of foods such as raw milk, cheeses (particularly soft-ripened varieties), raw and cooked poultry, raw meats (all types), and raw and smoked fish (Food and Drug Administration 2012), several outbreaks have been linked to the consumption of contaminated raw vegetables (Soriano

et al. 2001; Prazak *et al.* 2002; Thunberg *et al.* 2002; Dreux *et al.* 2007). Two documented outbreaks, which occurred in 1979 and 1981, respectively, were linked to salad vegetables (raw celery, lettuce and tomatoes) and cabbage (coleslaw) (Food and Drug Administration 2012). More recently, large outbreaks occurred in 2009 (contaminated sprouts), 2010 (diced celery), and in 2011 when 30 deaths and 142 hospitalizations were caused by an incidence of listeriosis linked to whole cantaloupes (Center for Disease Control and Prevention 2012).

Potential sources of contamination can be found at the preharvest stage, including soil, irrigation water, manure, air (dust), wild and domestic animals, human handling, and

water for other uses (e.g., pesticides, foliar treatments, growth hormones), and at the postharvest level, including wash and rinse water, human handling (workers, consumers), harvesting, sorting, packing, cutting and further processing equipment, packing materials, and transport containers and vehicles (Dreux *et al.* 2007; Food and Drug Administration 2012; Oliveira *et al.* 2012). Storage conditions are also crucial as after processing pathogens can survive and even grow on lettuce and other vegetables.

Survival and growth of pathogenic microorganisms varies by the type of the microorganism, the food and its storage conditions, such as temperature, time and relative humidity (RH) (Paull 1999; Tian *et al.* 2012). Therefore, temperature control is a crucial factor for the shelf life and quality maintenance of fruits and vegetables. *L. monocytogenes* is able to survive and grow under refrigeration temperatures (Koseki and Isobe 2005) and to survive and adapt to adverse environments (Gandhi and Chikindas 2007; Sant'Ana *et al.* 2012).

Another important factor for the shelf life and quality of vegetables is RH, which affects the rate of water loss from the plant tissues. However, the impact of RH is not independent of the temperature, as high RH can prevent moisture loss only if the product temperature is close to the air temperature (Paull 1999). Changing RH and water availability on plant surfaces can cause osmotic stress in epiphytic bacteria according to Brandl and Mandrell (2002), who reported that a decrease in RH from 100 to 60% caused a rapid reduction of *Salmonella enterica* population on cilantro leaves. Only limited information is available regarding the effect of RH and temperature on the survival of *L. monocytogenes* on vegetables, particularly on parsley and cucumber.

The objectives of this work were to investigate the effect of RH and storage temperature on the behavior of *L. monocytogenes* on lettuce, parsley and cucumber in order to improve the knowledge base that is relevant to food safety. The vegetables used in the study were selected to represent different surface structure and morphology: a broad flat leaf (lettuce); small, more complex leaf structure (parsley); and waxy cuticle (cucumber). Although many vegetables are usually refrigerated in the home or catering establishment, they may spend long periods in uncontrolled temperature conditions on market stalls, in greengrocers and during transport. For this study, the three temperatures of 10, 20 and 30°C were chosen to represent different climatic conditions to which unrefrigerated vegetables are exposed.

MATERIALS AND METHODS

Preparation of Vegetables

Romaine lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*, unwaxed) and flat-leaf parsley (*Petroselinum sativum*) were

purchased from a local greengrocer. Outer or damaged leaves and the core of the lettuce heads were removed and discarded. The inner leaves were cut in circular pieces ($r = 4.5$ cm) using a sharp sterile knife and each piece was placed in a sterile plastic Petri dish (Sarstedt AG & Co., Nuembrecht, Germany) with the upper (as the lettuce is opened out) leaf surface uppermost. For the cucumber, the skin was removed in strips with a sterile vegetable peeler. These strips were trimmed as required and laid outer side uppermost in a 90-mm Petri dish so that the whole dish area was covered with a single layer. The surface area of lettuce and cucumber samples was approximately 64 cm². The parsley leaves were cut with sterilized scissors and placed in Petri dishes in 2 g portions, distributed as evenly as possible with the upper leaf surfaces uppermost. The parsley was measured by mass as it was not possible to calculate the area of the small leaves with complex morphology.

Preparation of *L. monocytogenes* Inoculum

L. monocytogenes strains Scott A, NCTC 10527 and NCTC 11994 were grown for 24 h at 37°C in 10 mL tryptic soy broth (Merck, Darmstadt, Germany). Equal volumes of the three cultures were mixed together and washed three times by centrifuging (3,000 rcf, 15 min, Eppendorf, Hamburg, Germany, Centrifuge 5418) with quarter-strength (q-s) Ringer solution (Oxoid, Basingstoke, U.K.). Cell pellets were resuspended in q-s Ringer solution, with the final concentration being 10⁵–10⁶ cfu/mL.

Inoculation of Vegetables

A 0.1 mL aliquot of washed bacterial culture in q-s Ringer solution (10⁵–10⁶ cfu/mL) was spread over the upper surface of the lettuce and the parsley leaves and the external surface of the cucumber. For lettuce and cucumber, the surfaces were spot inoculated with six to eight droplets which were then spread around using the pipette tip in order for the entire surface to be covered as evenly as possible. The cut edges of the pieces were avoided. In the case of parsley, six to eight leaves were spot inoculated using droplets of sizes as close to equal as possible using the pipette tip. The inoculum was allowed 30-min contact time at room temperature before the samples were transferred to incubators at 10, 20, and 30°C, and 53 and 90% RH (Precision Instruments, ESP Inc., Kennesaw, GA). Samples were analyzed periodically over the experimental duration times of 7 days, 5 days and 24 h at 10, 20 and 30°C, respectively.

Enumeration of *L. monocytogenes*

Samples were placed in a stomacher bag (Seward Ltd., Worthing, U.K.) containing 50 mL q-s Ringer's solution and homogenized by mixing for 30 s (normal speed) with a

Bag-Mixer 400 stomacher (Seward Ltd.). For the enumeration of *L. monocytogenes*, 0.1 mL volumes of appropriate serial dilutions of the homogenate were spread on the surface of PALCAM agar plates (Merck), and the plates were incubated at 30C for 48 h. Uninoculated samples were also stored under the same conditions and analyzed for the presence of *L. monocytogenes*.

Data Analysis

For each group of experiments, two replicate experiments were conducted, with two samples evaluated per experiment, giving a total of four replicate samples. Microbial counts were transformed to logarithms before computing means and standard deviations; population densities were reported as log cfu/cm² for lettuce and cucumber samples, and as log cfu/g for parsley samples. Although the use of different units prevents direct comparison of absolute counts between parsley and the other vegetables, changes in log count can be compared as these are in effect ratios of populations on the same vegetable. Data from microbiological analyses were evaluated by analysis of variance using Minitab Statistical Software, Release 15 (Minitab Inc., State College, PA). The level of significance for all tests was 0.05.

RESULTS

The changes in the populations of *L. monocytogenes* on lettuce leaves, cucumber epidermis and parsley leaves stored at 10, 20, and 30C, and at 53 and 90% RH are presented in Figs. 1–3. On lettuce stored at 10C (Fig. 1, top), the population remained approximately constant at 90% RH for the duration of the experiment (an apparent decline at 5 days is likely to be an experimental artifact), while at 53% RH a steady decline was observed which reached approximately 1 log cfu/cm² reduction by day 7. At 20C (Fig. 1, middle), the *L. monocytogenes* population on lettuce declined by around 2 log cfu/cm² by day 4, although an increase of 0.5 log cfu/cm² was observed on day 5. There was no significant difference in the behavior of the *L. monocytogenes* populations stored at 53 and 90% RH. On lettuce stored at 30C (Fig. 1, bottom), the populations of *L. monocytogenes* remained approximately constant for 24 h and there was no significant difference between the lettuce samples stored at different RH.

On cucumber epidermis at 10C (Fig. 2, top), the population of *L. monocytogenes* remained relatively constant at 53% RH, but increased significantly at 90% RH after 3 days. The maximum population was observed at 4 days, an increase of 1.8 log cfu/cm² relative to the starting concentration, then a slow decline of 0.7 log cfu/cm² was recorded over the following 3-day period. At 20C (Fig. 2, middle), the population of *L. monocytogenes* on cucumber stored at 53%

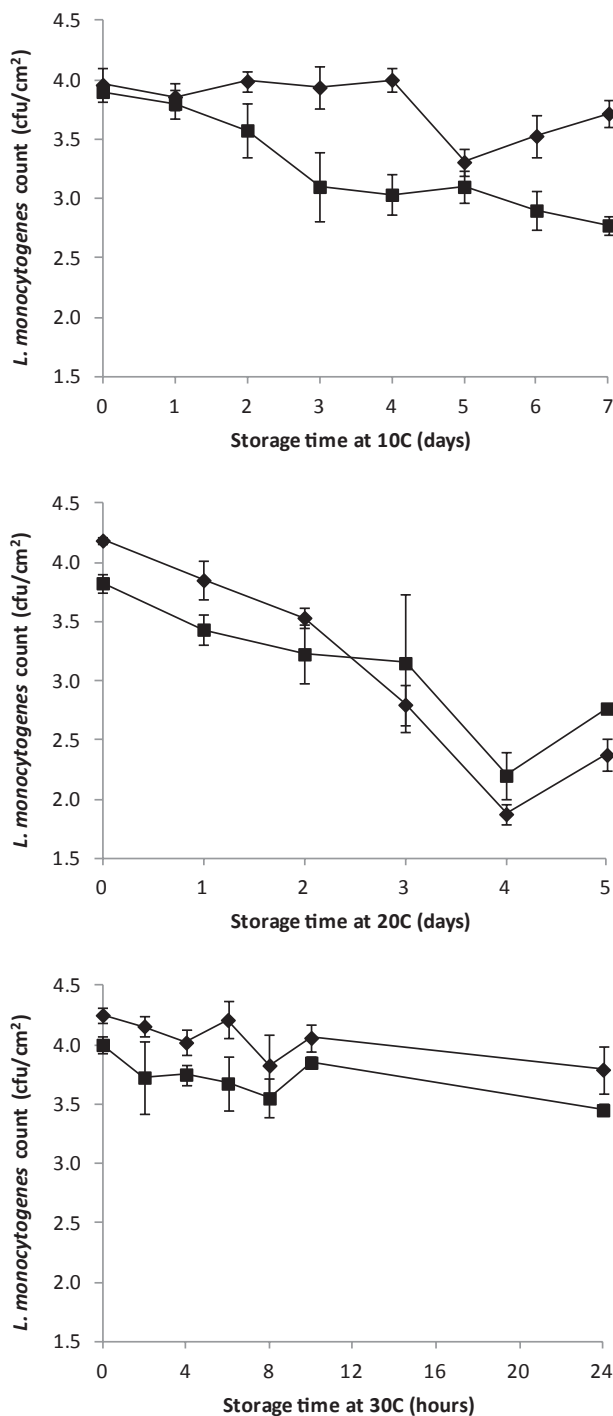


FIG 1. THE CHANGE IN THE POPULATION OF *LISTERIA MONOCYTOGENES* ON LETTUCE LEAVES STORED AT 10C (TOP), 20C (MIDDLE), AND 30C (BOTTOM), AND AT 53% (SQUARES) AND 90% (RHOMBI) RELATIVE HUMIDITY. ERROR BARS INDICATE 1 STANDARD DEVIATION FROM THE MEAN

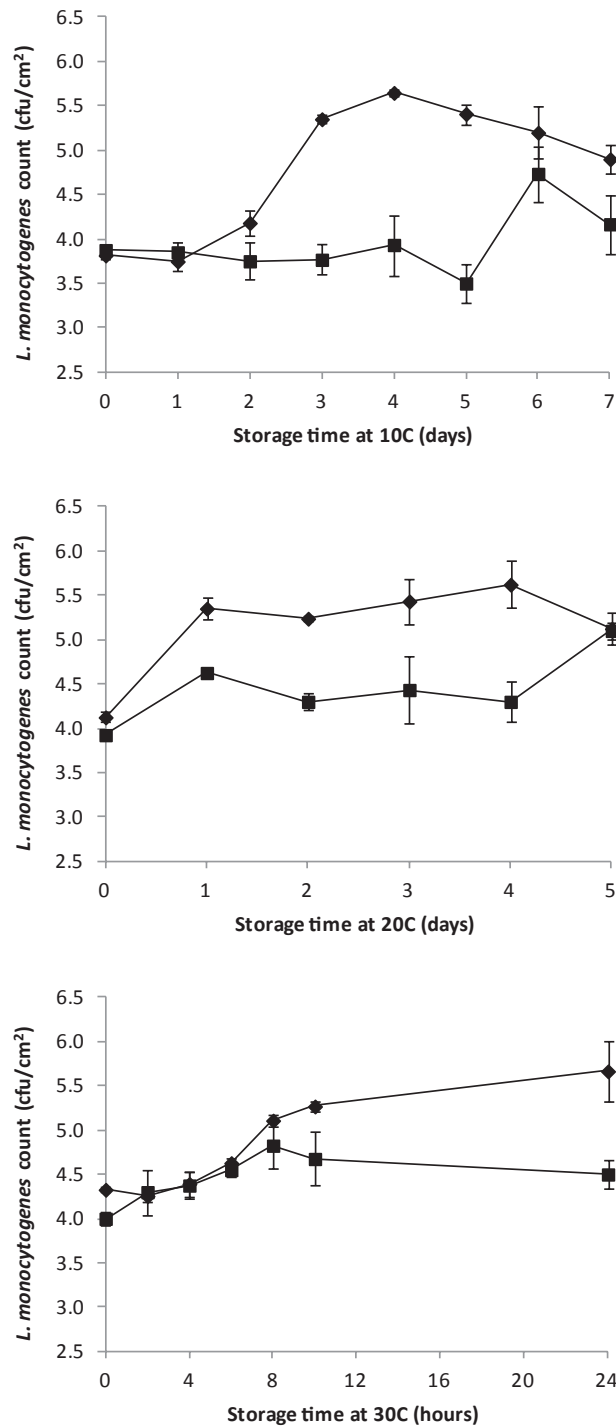


FIG 2. THE CHANGE IN THE POPULATION OF *LISTERIA MONOCYTOGENES* ON CUCUMBER EPIDERMIS STORED AT 10C (TOP), 20C (MIDDLE), AND 30C (BOTTOM), AND AT 53% (SQUARES) AND 90% (RHOMBI) RELATIVE HUMIDITY. ERROR BARS INDICATE 1 STANDARD DEVIATION FROM THE MEAN

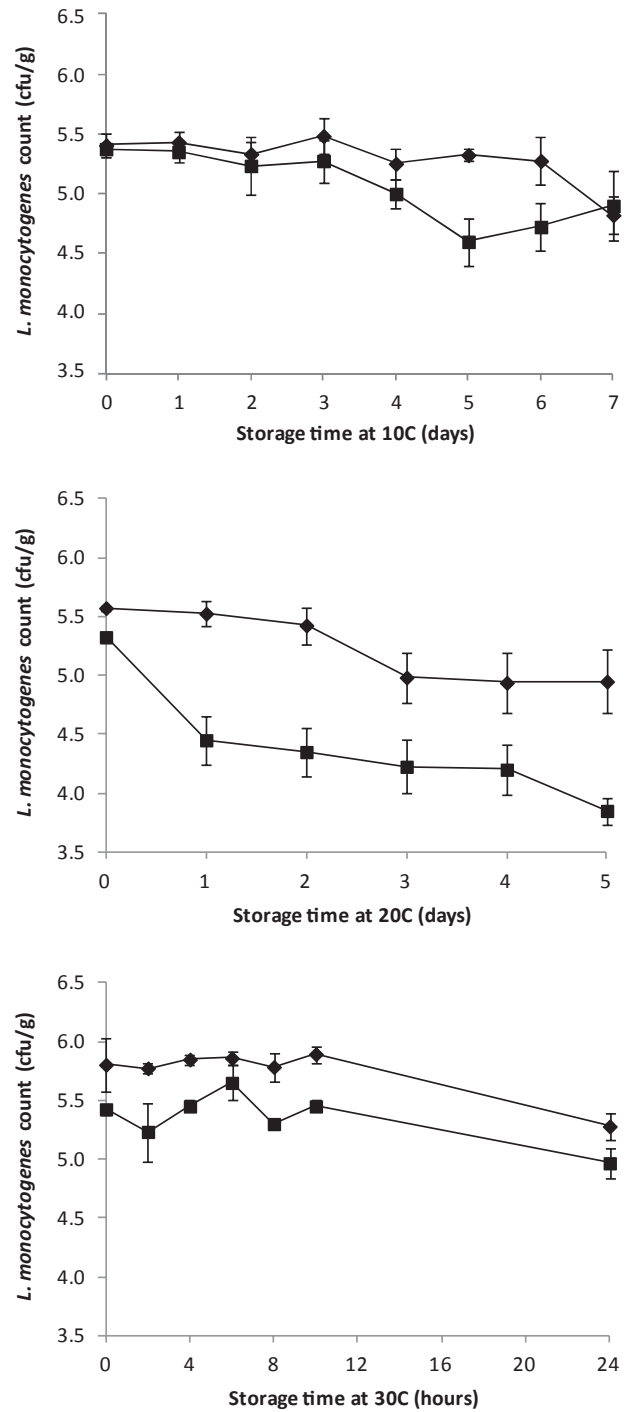


FIG 3. THE CHANGE IN THE POPULATION OF *LISTERIA MONOCYTOGENES* ON PARSLEY LEAVES STORED AT 10C (TOP), 20C (MIDDLE), AND 30C (BOTTOM), AND AT 53% (SQUARES) AND 90% (RHOMBI) RELATIVE HUMIDITY. ERROR BARS INDICATE 1 STANDARD DEVIATION FROM THE MEAN

RH increased only slightly ($0.7 \log \text{cfu/cm}^2$) after 1 day of storage then remained constant until day 5, at which time a further increase of $0.8 \log \text{cfu/cm}^2$ was observed. On cucumber stored at 90% RH, a larger initial increase ($1.3 \log \text{cfu/cm}^2$) was observed after 1 day followed by a period of stability until the end of the experiment at day 5. At time points 1–4 days, the population of *L. monocytogenes* on the cucumber stored at 90% RH was significantly ($P < 0.05$) higher than that on the cucumber stored at 53% RH, but there was no difference between the two on day 5.

L. monocytogenes inoculated onto parsley leaves and stored at 10C (Fig. 3, top) remained approximately at the starting level for the first 4 days at both RH values. On days 5 and 6, the population on leaves stored at 53% RH was significantly ($P < 0.05$) lower than that at 90% RH, but only by less than $1 \log \text{cfu/g}$. No difference was observed on day 7. The overall decline in population at 10C was minimal. At 20C (Fig. 3, middle) the population of *L. monocytogenes* on parsley leaves stored at 90% RH declined slowly by about $0.7 \log \text{cfu/g}$ over the 5 days of the experiment. At 53% RH, an initial decrease in count of $0.8 \log \text{cfu/g}$ was observed after 1 day of storage, and by the end of 5 days the total decrease was $1.4 \log \text{cfu/g}$. At every time point after day 0, the *L. monocytogenes* count on leaves stored at 53% RH was significantly ($P < 0.05$) lower than that on leaves stored at 90% RH. At 30C (Fig. 3, bottom), the *L. monocytogenes* populations remain at approximately the same level for the duration of the experiment. Although there are significant differences between the counts at 90 and 53% RH, this is an artifact caused by a difference in the starting count.

Presumptive *L. monocytogenes* colonies were not isolated from any of the uninoculated samples.

DISCUSSION

Growth of *L. monocytogenes* was observed on cucumber epidermis, but not on either of the leafy vegetables. The reasons for this are not entirely clear, but could be due to differences in nutrient availability on the surfaces of the different vegetables. The lack of growth on lettuce contrasts with findings of other authors such as Carrasco *et al.* (2008), Tian *et al.* (2012) and Sant'Ana *et al.* (2012). However, these authors cited all used shredded or cut lettuce in their experiments, so the *L. monocytogenes* had access to nutrients and water leaking from the cut surfaces. In our experiments, the bacteria were inoculated onto a leaf surface that was undamaged (as far as could be determined by visual inspection). All (10 and 20C) or most (30C) of the growth of *L. monocytogenes* on cucumber occurred prior to the end of the incubation period, indicating that a population greater than approximately $5.5 \log \text{cfu/cm}^2$ would not be obtained with extended incubation. This could be because this figure represents the carrying capacity of the

cucumber epidermis with respect to *L. monocytogenes*, or because the conditions on the epidermal surface became unfavorable to growth prior to the end of the incubation time, perhaps due to loss of water from the tissues. *L. monocytogenes* increased by around $1 \log \text{cfu/cm}^2$ within 10 h on cucumber stored at 30C and 90% RH, conditions that could be encountered at a vegetable market in a country with a warm climate, for example. At 20C (e.g., representing storage outside the refrigerator in the home), an increase of over $1 \log \text{cfu/cm}^2$ was observed after 1 day at 90% RH. Lowering the temperature to 10C extended the time required for the maximum population to be reached to 4 days.

The ability of *L. monocytogenes* to grow on cucumber (rather than simply survive) could significantly affect the probability of illness in the consumer. While the infectious dose of *L. monocytogenes* in healthy individuals is somewhat uncertain, it is generally considered to be fairly high (McLauchlin *et al.* 2004). Contamination of vegetables by *L. monocytogenes* is not uncommon (Beuchat 1996; Francis and O'Beirne 2005), but the viable counts are usually low. A meta-analysis by Crépet *et al.* (2007) of studies of unprocessed or minimally processed vegetables revealed a mean *L. monocytogenes* concentration of $-2.63 \log \text{cfu/g}$ on contaminated vegetables, and the probability of viable counts higher than 1, 2 and 3 $\log \text{cfu/g}$ was 1.44, 0.63 and 0.17%, respectively. The ability to grow on the vegetable surface may therefore increase the numbers of *L. monocytogenes* from a level that would be harmless to most consumers to one which could potentially cause illness.

Our data indicate that RH is a more important factor for survival of *L. monocytogenes* at cooler storage temperatures. Similarly, Tian *et al.* (2013) reported that reduction levels of *Escherichia coli* O157:H7 and *Salmonella typhimurium* on unblemished and bruised apple surfaces were higher at RH of 85 and 68% than at RH of 100% at 15C.

In the current paper, viable bacterial counts were conducted using a culture-dependent method (colony counting on selective media). Dreux *et al.* (2007) conducted an experiment in which *L. monocytogenes* was inoculated onto the leaves of growing parsley plants at low variable ambient humidity (47–69%). Their survival was monitored using plate counting and a culture-independent method (BacLight, and two others). Over 15 days, a decline in colony count from approximately 9 to around 2 $\log \text{cfu/leaf}$ was observed, but the decline in count using BacLight was only around 6 $\log \text{cfu/leaf}$, most of which occurred in the first 2 days. This suggests that some of the bacteria entered a viable but nonculturable (VNC) state in response to the stress caused by low humidity. It is possible that a similar phenomenon occurred in our experiment and that the apparent decreases in count are larger than the actual decreases in number of viable cells. However, in Dreux *et al.*

(2007), the proportion of the *L. monocytogenes* cells that apparently entered a VNC state was small (around 0.1%), and so unlikely to have a significant impact on product safety, especially given the normally low initial numbers (Crépet *et al.* 2007).

Growth of *L. monocytogenes* on cucumber epidermis was also affected by RH, the population increasing by up to 1 log cfu/cm² more on the samples stored under 90% RH. RH is therefore an important factor in the safety of fresh vegetables as far as *L. monocytogenes* is concerned. However, the extent to which RH can be controlled and reduced is often limited. Even if the air in a storage facility has a reduced RH, local increases could occur next to the surfaces of those vegetables in the middle of a pile or box. It is also likely that a conflict would arise between controlling microbial growth and maintaining the physical quality of the vegetables by limiting water loss. According to Paull (1999), the recommended temperature and RH for storage of cucumber to maintain product quality are 10°C and 95%, respectively, but *L. monocytogenes* was found to grow under similar conditions (Fig. 2, top). Special care should therefore be taken in washing cucumbers prior to consumption, especially if they are intended for vulnerable groups such as hospital patients and given that *L. monocytogenes* is not easily removed from cucumber epidermis by simple washing (Nastou *et al.* 2012).

L. monocytogenes was able to grow on cucumber epidermis, but not on the undamaged leaf surfaces of lettuce and parsley. This is an important consideration when comparing our data with that of other authors, most of which use shredded/cut leaves on which growth is often observed. The RH under which fresh vegetables are stored is an important factor affecting the growth or survival of *L. monocytogenes* on the leaf or fruit surface. Low RH decreases the growth rate and increases the death rate of the organism, thereby improving food safety, but this consideration needs to be balanced with that of maintaining product quality. While temperature and RH control is obviously not possible for vegetables in the field, where contamination with *L. monocytogenes* may occur, it may comprise a safety factor in the case of postharvest cross-contamination and growth. The effect of RH on growth or survival was greater at cooler temperatures.

ACKNOWLEDGMENT

This work was funded internally by the Research Committee of Alexandrian Technological Educational Institute of Thessaloniki. No outside funding was used.

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