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Osmotic Dehydration of Apple Slices with $CaCl_2$ and Sucrose Limits Decay Caused by *Penicillium expansum, Colletotrichum acutatum,* and *Botrytis cinerea* and Does Not Promote *Listeria monocytogenes* or Total Aerobic Population Growth

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ABSTRACT

The interaction of *Penicillium expansum* Link, *Colletotrichum acutatum,* and *Botrytis cinerea* Pers.:Fr. with *Listeria monocytogenes* on osmotically dehydrated apple slices was evaluated. In mineral analyses of the slices, the calcium content of the peel and flesh tissues increased by 4- and 11-fold, respectively, when processed in 2% $CaCl_2$. These slices also exhibited less decay by *P. expansum, C. acutatum,* and *B. cinerea.* Inoculation of slices with *P. expansum* resulted in a decrease in the pH of the flesh tissue at the infection site, while the pHs of slices infected with *C. acutatum* and *B. cinerea* increased and remained stable, respectively. Total mold population increased in wounds inoculated with *P. expansum* or *C. acutatum.* The presence of *L. monocytogenes* in the wounds did not significantly affect mold growth. The association of *P. expansum* and *L. monocytogenes* on apple slices resulted in a decrease in the bacterial population, whereas *L. monocytogenes* survived when slices were inoculated with *C. acutatum.* When associated with *B. cinerea,* there was a fourfold decrease in the *L. monocytogenes* population when slices were treated with 2% $CaCl_2$. The total aerobic population was not significantly affected by the type of microorganism added to the wounds or by the osmotic treatment. These data show that osmotic dehydration with 2% $CaCl_2$ combined with 20% sucrose limits decay of apple slices and does not promote bacterial or total aerobic population growth.

There has been a net increase in fresh fruit consumption during the last decade in the United States due to consumer interest in a healthy and nutritious diet. Processed produce with the qualities (flavor, texture, appearance) of fresh produce are gaining popularity. On the other hand, the number of human pathogen–related illnesses reported after consumption of fresh or lightly processed produce has increased *(5).* The major challenge for the food industry is to offer quality produce that is safe *(10).* The increase in salad bars and consumer preference for organic produce necessitate research aimed at reducing the risks of outbreaks due to produce contamination. *Listeria monocytogenes* is a major concern. Each year, this bacterium causes an estimated 2,500 cases of listeriosis, 500 of which are fatal *(6).* The vast majority of these outbreaks have resulted in the recall of products of mainly animal origin. Outbreaks from produce were not a major concern because the incidence of *Listeriae* in produce destined for human consumption was not clearly related to human listeriosis. Additionally, raw produce is supposedly free from enteric pathogens unless human and/or animal waste or contaminated irrigation water is used. Therefore, many produce-related outbreaks have gone unreported. However, the increase in the amount of lightly processed produce marketed has resulted in an increase in produce recalls such as the recent recall of sliced-apple packages distributed in 17 states from the Pacific Northwest *(17).*

Unlike the situation for raw vegetables, there is virtually no information concerning the incidence and survival of *Listeria* spp. on raw and lightly processed fruit. Contamination of fresh and lightly processed fruit can originate from multiple sources. Increased exposed surface area in sliced fruit results in higher risks for contamination. Unlike processed juice, there is no terminal critical point for fresh or lightly processed produce. Metabiotic association of molds and *Clostridium botulinum* on tomatoes *(11)* and of molds and *L. monocytogenes* on fresh-cut apples *(8)* has been reported. Factors such as produce conditions during storage and transport affect the potential for causing illness. Calcium treatment was effective in maintaining fruit quality during postharvest storage life *(15)* and decreased susceptibility to decay caused by *Botrytis cinerea, Glomerella cingulata,* and *Penicillium expansum (9).* Osmotic dehydration of produce with concentrated sucrose solution has been effective in partially removing water from the plant tissue and therefore increasing the shelf life of produce. When combined with different $CaCl_2$ concentrations, the osmotic

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treatment that was most effective in reducing decay caused by *P. expansum, Colletotrichum acutatum,* and *B. cinerea* was 2% $CaCl_2$ plus 20% sucrose *(7).*

Decay caused by fungal plant pathogens often results in changes in plant tissue characteristics that might favor bacterial development. Therefore, the objectives of this work were to determine the effects of osmotic dehydration using sucrose and $CaCl_2$ on fungal decay caused by *P. expansum, C. acutatum,* and *B. cinerea* and to investigate the association of these plant pathogens with *L. monocytogenes* on lightly processed apple slices.

MATERIALS AND METHODS

Apple fruits. Golden Delicious apples purchased at a local orchard were stored at 4°C until needed. Fruits were sorted (7.5 ± 0.7 cm diameter and 7.4 ± 0.3 cm height), and firmness was measured for 15 fruits with a manually controlled Instron Effe-gi (McCormick, Yakima, Wash.) with a 1-cm-diameter tip penetrating to a depth of 25.4 mm. Firmness (43 ± 3.7 N) was measured at two opposite sites on the equator of the fruit after removal of a 2-mm tissue slice with a fixed-blade slicer. Refractive index (1.3527 ± 0.002) and °Brix (13.13 ± 1.15) were measured on the slurry of the same 15 fruits using a refractometer. Fruits were surface-sterilized with 70% ethanol and sliced into eight equal slices, and the cores were removed. Slices of three selected apples were combined, randomly divided into five lots of four slices, and immediately used for osmotic dehydration.

Osmotic dehydration. Nonreagent grade sucrose (Kroger, Cincinnati, Ohio) and $CaCl_2 \cdot 2H_2O$ (Fischer, Fair Lawn, N.J.) were used to prepare the osmotic solutions in tap water, and treatments with 0% $CaCl_2$ plus 0% sucrose (water control), 0% $CaCl_2$ plus 20% sucrose, 2% $CaCl_2$ plus 0% sucrose, and 2% $CaCl_2$ plus 20% sucrose were tested, along with untreated control slices. Each lot of four slices was placed in a 2-liter Pyrex beaker containing 1 liter of solution. The slices were covered with a stainless grip cage for full immersion in the solutions. The mass ratio of product to solution was at least 1:10. A small submersible electric pump was placed in each beaker during the osmosis process to ensure a uniform sugar concentration and a uniform temperature around the samples. The temperature-controlled water bath held up to four beakers. Osmotic dehydration was performed at 25°C for 1 h. Untreated control slices were not subjected to osmotic dehydration.

Organisms and preparation of inocula. *B. cinerea* originally isolated from apple fruit was acquired from Dr. Wojciech J. Janisiewicz, Agricultural Research Service, USDA, Appalachian Fruit Research Station, Kearneyville, W. Va. The fungus was grown on fresh potato dextrose agar (PDA; Difco Laboratories, Detroit, Mich.) prepared as follows: 300 g of sliced potatoes added to 500 ml of deionized water was boiled until thoroughly cooked and then filtered through cheesecloth, and water was added to the filtrate to a final volume of 1 liter. Fifteen grams of agar and 20 g of glucose were added to the filtrate before autoclaving. *P. expansum* (originally recovered from a decayed apple in storage) and *C. acutatum* (obtained from Dr. Kenneth D. Hickey, Penn State Fruit Lab and Extension Center, Biglerville, Pa.) were both grown on PDA. All plant pathogen cultures were grown for a minimum of 6 days at 20°C under constant fluorescent light before use. Conidia were harvested and conidial suspensions prepared in deionized water containing 0.2% (wt/vol) Tween 20 (Sigma Chemical, St. Louis, Mo.) were adjusted to 10^6 conidia per ml with a hemacytometer. The Scott A strain of *L. monocytogenes* (ATCC 7644) was cultured on tryptic soy broth (TSB, pH 7.3 at 30°C; Difco). Two consecutive 48-h transfers and a final 24-h transfer at 30°C were made by placing a 1-ml aliquot of the suspension in 5 ml of TSB. The tryptic soy agar (TSA; Difco) slants were loop-inoculated, and after a 24-h transfer at 30°C, the slants were used to loop-inoculate 10 ml of TSB. After incubation at 30°C for 24 h, the bacterial suspension was centrifuged (2,000 × *g,* 10 min, 4°C) and the pellet was washed twice in peptone water. The bacterial suspension (about 25 × 10^8 CFU/ml in peptone water) was serially diluted in peptone water, and dilutions were surface-plated on TSA to determine the exact bacterial count.

Inoculation. Slices were removed from the sucrose solutions, dipped for 2 s in sterile deionized water, and placed on a screen for 20 min to drain excess osmotic solution. Two wounds were made on each slice to a depth of 6 mm with the tip of a disinfected tenpenny nail approximately 4 mm in diameter at the base as described by Conway et al. *(8).* One wound was made at one end of the slice, and a second one was made in the center of the slice. Both wounds were inoculated with 20 μl of a conidial suspension of *P. expansum, C. acutatum,* or *B. cinerea.* Inoculated slices were individually placed in small plastic bags (Ziploc, Dow Brands, Indianapolis, Ind.) and incubated at room temperature under constant light. The severity of decay was recorded 48 h after inoculation by measuring the diameter of the decayed area as the mean of its width and length and computing the total area of decay. Control slices were inoculated with 20 μl of water containing 0.2% Tween 20. Two days after mold inoculation, 20 μl of *L. monocytogenes* suspension was inoculated in the central wound and in a third, freshly made, wound (at the site opposite that of the first wound) of the apple slice. Slices were placed in plastic bags and incubated at room temperature under constant light for 24 h.

pH measurement. The pH of the apple tissue inside and 5 mm outside the wound site was measured with a pH/ion analyzer 350 (Corning, Corning, N.Y.) on the third day after inoculation with the fungal plant pathogens.

Microbial analyses. Inoculated slices were cut into three pieces for microbial recovery of mold, *L. monocytogenes,* and the total aerobic population. Each apple piece (one wound per piece) was placed in a whirlpack bag containing 8 ml of Listeria enrichment broth (LEB; Difco). After homogenization, the pH of the mixture was adjusted to 6.5 to 7 before incubation at 30°C for 2 h. The homogenate was serially diluted in peptone water, and 100-μl aliquots were surface-plated on modified Oxford agar (MOX; Difco) for the *L. monocytogenes* count, on Rose Bengal agar (RBA; Difco) for the total mold count, and on TSA for determination of the total aerobic population. Plates were incubated at 30°C for MOX and TSA and at room temperature for RBA. The number of colonies formed was recorded after 48 h and is expressed as $\log_{10}$ CFU/ml in LEB.

Calcium measurement. Peel and flesh tissues of untreated control slices and slices inoculated with only mold were freeze-dried separately and ground, and samples were kept at 60°C until dry weight was constant. Samples consisting of 500 mg of dried flesh tissue and 200 mg of peel tissue were ashed (500°C, 12 h) and suspended in 2 N HCl before analysis by inductively coupled argon plasma atomic emission spectrometry.

Statistical analysis. The experimental design was an incomplete block design. Each lot of four slices represented one replicate composed of one uninoculated slice and three slices inoculated with *P. expansum, C. acutatum* or *B. cinerea,* and *L. mon-*

TABLE 1. *Calcium content of apple tissue as affected by osmotic dehydration in $CaCl_2$-plus-sucrose solutions for 1 h at 25°C*

Treatment (% $CaCl_2$, % sucrose)	Calcium content (μg/g dry wt)	
	Peel	Flesh
Untreated control	674 B[a]	283 C
0, 0	657 B	391 C
0, 20	560 B	307 C
2, 0	2,675 A	3,187 B
2, 20	2,617 A	3,906 A

[a] Data are means of nine replications. Means within a column followed by the same letter are not significantly different based on Duncan's multiple-range test ($P < 0.05$).

ocytogenes. The experiment was repeated three times. Data presented are means of at least three replicates. Data were compared using Duncan's multiple-range test at $P < 0.05$ *(16)* unless otherwise indicated.

RESULTS

Calcium content. Osmotic dehydration of apple slices in solutions containing $CaCl_2$ resulted in a fourfold increase in the Ca content of the apple peel compared with the untreated control, and this accumulation was not affected by the combination of $CaCl_2$ and sucrose (Table 1). The calcium content of the apple flesh showed an 11-fold increase compared with the control slices when slices were processed in 2% $CaCl_2$ plus 0% sucrose. The combination of 20% sucrose and 2% $CaCl_2$ resulted in an additional 22% increase in the Ca content of the apple flesh.

Decay development. Slices infected with *P. expansum* exhibited maximum decay when processed in water (Table 2). Osmotic dehydration in 20% sucrose resulted in a 39% decrease in the decay area caused by *P. expansum* compared with the untreated control. Treatments with 2% $CaCl_2$ alone or in combination with 20% sucrose limited decay by 55%. Decay by *C. acutatum* was reduced by 55 and 40% when slices were processed in solutions containing 2% $CaCl_2$ with or without sucrose, respectively. Osmotic dehydration performed with 2% $CaCl_2$ alone or in combination with 20% sucrose decreased decay caused by *B. cinerea* by 31 and 37%, respectively, compared with the untreated slices. Uninoculated slices did not exhibit any noticeable fungal decay.

Changes in pH. The pH inside the wound infected with *P. expansum* decreased, regardless of the treatment, as $\Delta pH > 0$ (ΔpH is the pH of the tissue 5 mm outside the wound minus the pH of the tissue inside the wound) (Fig. 1). Solutions containing 20% sucrose alone limited this decrease by 40% compared with the untreated control. Infection of apple slices with *C. acutatum* resulted in an increase in the pH of the tissue at the wound site ($\Delta pH < 0$), except when slices were processed in a solution containing 0% $CaCl_2$ and 20% sucrose. Decay by *B. cinerea* did not result in any significant variation in the pH of the tissue. The pH of the tissue outside the wound was compared on slices infected with each fungus (not shown). When a wound was

TABLE 2. *Decay area (mm^2) caused by* P. expansum, C. acutatum, *and* B. cinerea *on apple slices as affected by osmotic dehydration in $CaCl_2$-plus-sucrose solutions for 1 h at 25°C*

Treatment (% $CaCl_2$, % sucrose)	Fungal pathogen		
	P. expansum	*C. acutatum*	*B. cinerea*
Untreated control	61.70 B[a]	44.13 A	34.12 A
0, 0	74.60 A	45.56 A	40.01 A
0, 20	43.54 C	48.35 A	37.55 A
2, 0	27.85 D	19.88 B	23.53 B
2, 20	27.79 D	25.39 B	21.37 B

[a] Data are means of nine replications. Means within a column followed by the same letter are not significantly different based on Duncan's multiple-range test ($P < 0.05$).

inoculated with *C. acutatum,* the pH of the tissue outside the wound was at least 1 pH unit greater than that of the tissue outside the wounds inoculated with *P. expansum* or *B. cinerea.*

Enumeration of microorganisms. Wounds inoculated with *P. expansum* showed a 60 to 70% increase in total mold population after 3 days (Fig. 2). This increase was not affected by the presence of *L. monocytogenes.* No significant growth of the *L. monocytogenes* population was observed in wounds inoculated with the bacterium only. When a wound was inoculated with both *P. expansum* and *L. monocytogenes,* the bacterial population decreased. The total aerobic population was not affected by the treatment or by the type of microorganism with which a wound was inoculated. The total mold population increased by up to 80% in wounds inoculated with *C. acutatum,* and fungal growth was not affected by the presence of *L. monocyto-*

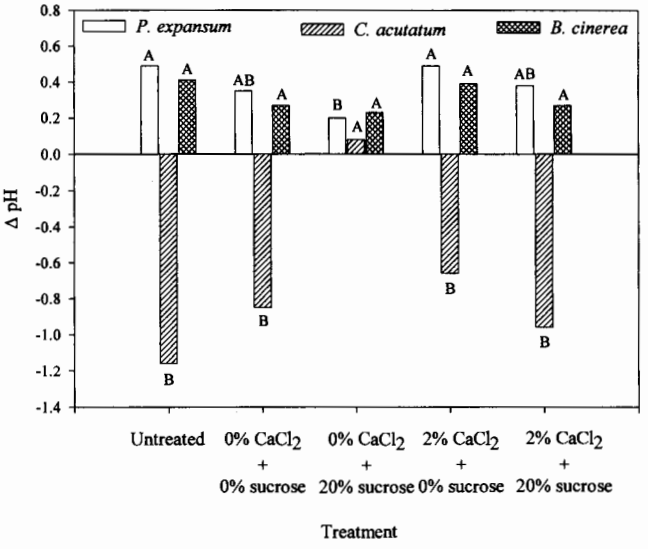

FIGURE 1. *Effect of* P. expansum, C. acutatum, *and* B. cinerea *on the pH of apple slices processed in $CaCl_2$-plus-sucrose solutions for 1 h at 25°C. The pH was recorded 3 days after inoculation. ΔpH is the pH of the tissue 5 mm outside the wound minus the pH of the tissue inside the wound. Data are means of three replications. For each fungus, bars with the same letter are not significantly different on the basis of Duncan's multiple-range test ($P < 0.05$).*

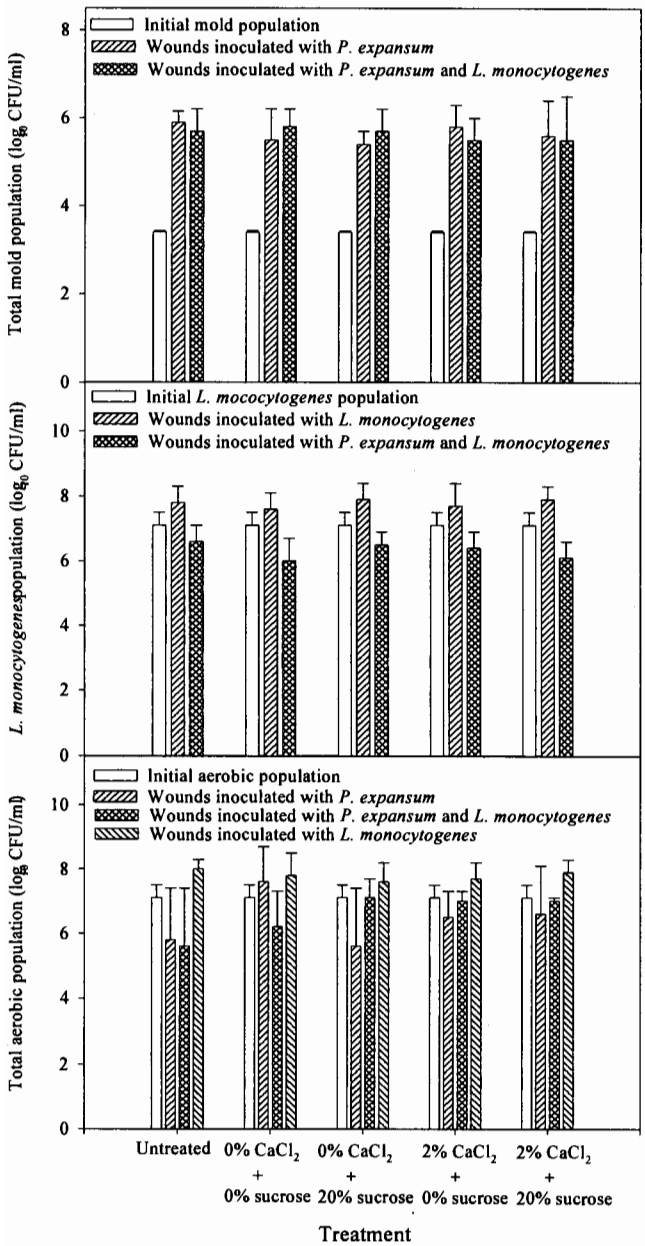

FIGURE 2. *Effect of osmotic dehydration in $CaCl_2$-plus-sucrose solutions on microbial populations of apple slices infected with* P. expansum *and* L. monocytogenes. *Slices were processed for 1 h at 25°C, wounded, and inoculated with* P. expansum *2 days prior to inoculation with* L. monocytogenes. *Initial populations were determined following inoculation. Microbial analyses were performed 24 h after* L. monocytogenes *inoculation. The bars represent the standard deviation of the mean of three replicates.*

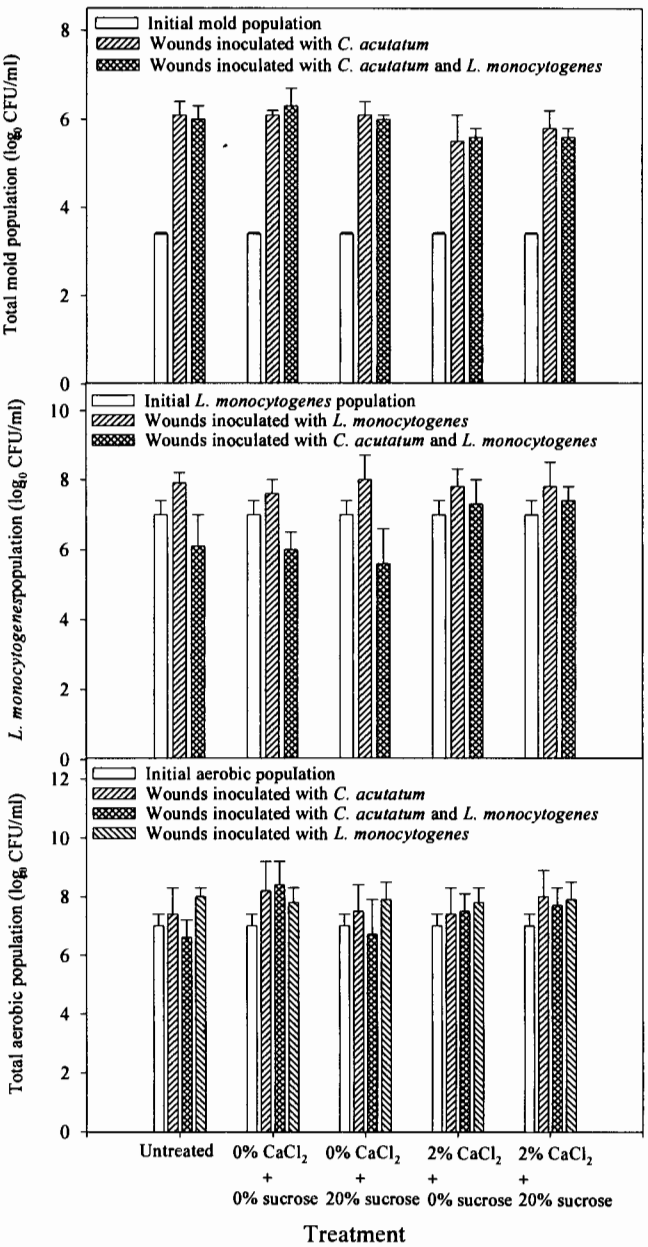

FIGURE 3. *Effect of osmotic dehydration in $CaCl_2$-plus-sucrose solutions on microbiological populations of apple slices infected with* C. acutatum *and* L. monocytogenes. *Slices were processed for 1 h at 25°C, wounded, and inoculated with* C. acutatum *2 days prior to inoculation with* L. monocytogenes. *Initial populations were determined following inoculation. Microbial analyses were performed 24 h after* L. monocytogenes *inoculation. The bars represent the standard deviation of the mean of three replicates.*

genes in a wound (Fig. 3). *Listeria* populations remained stable in wounds inoculated with the bacterium only. When associated with *C. acutatum, L. monocytogenes* populations decreased on slices treated with water. The total aerobic count was not affected by the treatment or by the microorganism with which a wound was inoculated. The total mold population of the untreated slice decreased in wounds inoculated with *B. cinerea* alone and increased in slices treated with water and 20% sucrose (Fig. 4). The presence of *L. monocytogenes* did not affect the growth of *B. cinerea. Listeria* populations increased by 7 to 10% in wounds inoculated with the bacterium only. The association of *B. cinerea* and *L. monocytogenes* in the same wound did not promote the growth of *L. monocytogenes,* and the *Listeria* population decreased up to fourfold when slices were treated with 2% $CaCl_2$ alone. The total aerobic population increased by 9 (slices treated with water or 2% $CaCl_2$) to 15% (slices treated with 20% sucrose) in wounds inoculated with the bacteria only. A decrease in aerobic population was seen in the wounds inoculated with *B. cinerea* alone or with *L. monocytogenes.* The association of *B. cinerea* and *L. monocytogenes* on slices treated with 2% $CaCl_2$

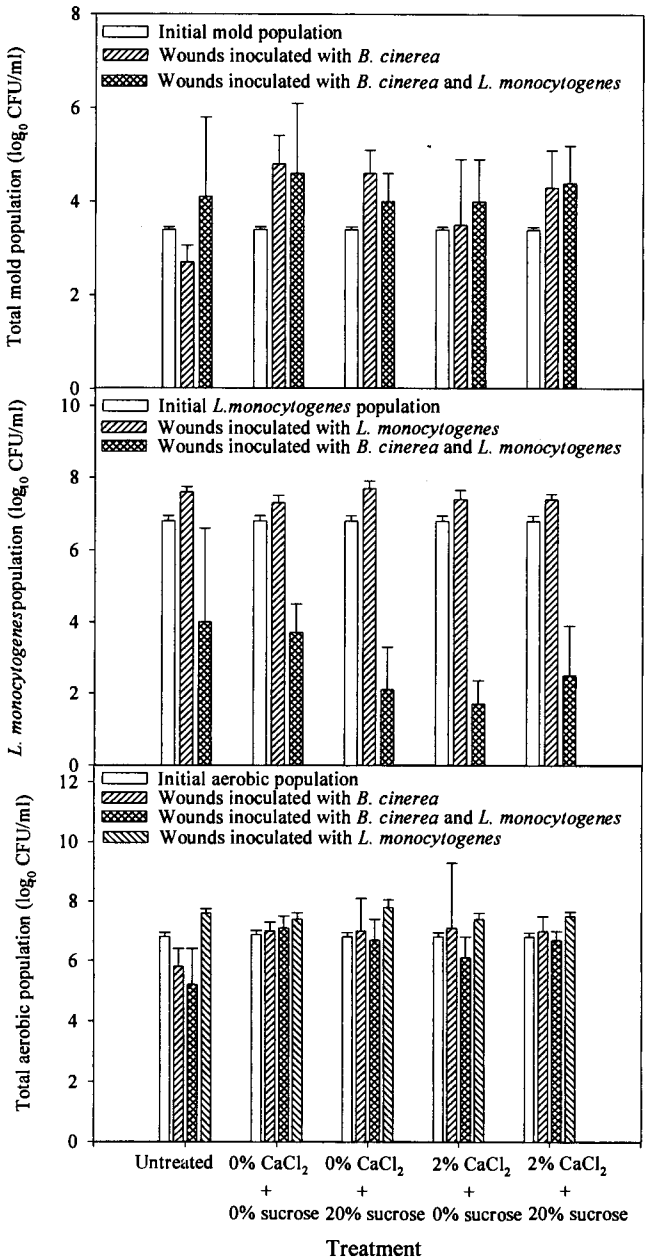

FIGURE 4. *Effect of osmotic dehydration in $CaCl_2$-plus-sucrose solutions on microbiological populations of apple slices infected with* B. cinerea *and* L. monocytogenes. *Slices were processed for 1 h at 25°C, wounded, and inoculated with* B. cinerea *2 days prior to inoculation with* L. monocytogenes. *Initial populations were determined following inoculation. Microbial analyses were performed 24 h after* L. monocytogenes *inoculation. The bars represent the standard deviation of the mean of three replicates.*

alone or in combination with sucrose did not result in aerobic population growth.

DISCUSSION

Ready-to-use produce and fresh-cut fruits such as apples for salad bars are becoming more popular *(4)*. Compared with the whole fruit, the cut surfaces and the resulting leakage from fresh-cut fruit tissue can supply the nutrients necessary for microbial growth; consequently, the potential for contamination increases. The techniques used for lightly processing produce are generally not sufficient to ensure sterility or microbiological stability. The peel is a protective physical barrier against decay, and cut-up pieces are therefore more susceptible to fungal infection. The metabiotic association between molds and bacteria on produce has already been reported *(11)*. Decay by *Fusarium, Alternaria,* and *Rhizoctonia* resulted in an increase in the pH of infected tomato tissue, thus promoting the growth of *C. botulinum.* Fruits such as apples are usually acidic and do not support the growth of human pathogens like *Listeria.* Our data show that the pH of the apple slices was not significantly affected by *B. cinerea* decay, while *P. expansum* and *C. acutatum* decreased and increased the pH of the slices, respectively. In previous experiments, the association of *P. expansum* or *G. cingulata* with *L. monocytogenes* on apple slices resulted in more extensive pH variation 2 days after inoculation when both human and plant pathogens were inoculated at the same concentration or with 1 log difference *(8)*. Our data show that the decrease in the pH of the tissue infected by *P. expansum* did not promote the growth of *L. monocytogenes,* and the bacterial population decreased. The increase in pH caused by *C. acutatum* decay only allowed the survival of *L. monocytogenes.* The increase in pH has been shown to be beneficial for other bacteria. Riordan et al. *(13)* showed that the growth of *E. coli* O157:H7 was related to an increase of 2 pH units in the apple tissue caused by *G. cingulata.* The pH changes in the apple slices infected with *C. acutatum* were at least 1 pH unit greater than those of the slices infected with *P. expansum* and *B. cinerea.* We also found that the pH outside the wound inoculated with *C. acutatum* was greater than that of the tissue outside the wounds inoculated with *P. expansum* or *B. cinerea.* This suggests that the apparently noninfected tissue area was infected by *C. acutatum* and that the changes in the pH of the tissue infected by *C. acutatum* might have been underestimated. Decay caused by *B. cinerea* did not lead to a significant change in the pH of the tissue, and when associated with *L. monocytogenes,* the bacterial population decreased markedly. Therefore, bacterial death might be due not only to the low pH naturally occurring in the fruit, but also to compounds induced by or synthesized by *B. cinerea* that would have an antilisterial property. For example, the bactericidal effects of carrot juice on *L. monocytogenes* have been attributed to phytoalexines *(3)*.

Osmotic dehydration of apple slices in solutions containing 2% $CaCl_2$ greatly reduced decay, and these data are in agreement with a previous study *(7)*. The effects of calcium on decay development and on total mold population might appear contradictory. Fungal mycelium was primarily responsible for decay development, while conidia were mostly considered in CFU counts for total mold determination. Consequently, comparisons between the two determinations may not be valid. The development of plant pathogens on produce has been associated with toxins that can be released within the fruit and can be found in the healthy tissue surrounding the decayed area, thus causing illnesses *(11)*. The effectiveness of $CaCl_2$ in controlling *P. expansum, G. cingulata,* and *B. cinerea* has already been

reported for whole apples *(9)*. Our data show that $CaCl_2$ is also effective in protecting the exposed flesh tissue from mycelial development on apple slices. Slices processed in 2% $CaCl_2$ solutions alone or in combination with sucrose resulted in flesh tissue containing 11-fold more calcium than the untreated flesh tissue. Consequently, decay by all three plant pathogens was greatly reduced. The calcium concentration of the tissue 3 mm under the peel was approximately 1,500 $\mu g \cdot g^{-1}$ dry weight tissue in apples infiltrated with 2% $CaCl_2$ *(1)*. Sensory panels that have evaluated the effect of calcium treatment on the sensory flavor and texture of apples have shown that the overall acceptability of apples treated with 2% $CaCl_2$ is not affected *(2)*. We found that the calcium content of apple slices treated with 2% $CaCl_2$ was at least twice that of the Ca-treated whole fruit. Sensory analyses should be performed to determine whether the combination of sucrose and $CaCl_2$ counteracted the effect of high calcium content on the flavor of the apple slices. NaCl has been extensively used for vegetable preservation, and concentrations above 5% affect the viability of *L. monocytogenes (5)*. The use of plastic bags and the rapid respiratory activity of sliced plant tissue have been shown to generate a high-CO_2 atmosphere *(14)*, and Gram positive bacteria such as *L. monocytogenes* can grow in both aerobic and anaerobic environments *(12)*. The CO_2 atmosphere inside the plastic bag used in our study allowed the survival of *L. monocytogenes*. The total aerobic population was not significantly affected by the osmotic dehydration in $CaCl_2$, sucrose, or $CaCl_2$-plus-sucrose solutions.

Our data suggest that the osmotic dehydration of apple slices in solutions containing $CaCl_2$ combined with sucrose does not promote bacterial growth. In addition to its role in limiting decay, calcium may be a compound of choice for lightly processed fruit because of its nutritional value.

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