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Survival of *Salmonella* on Lemon and Lime Slices and Subsequent Transfer to Beverages

ABSTRACT

Little is known about the microbial risks associated with adding lemon and lime slices to beverages in the foodservice industry. *Salmonella* survival on lemon and limes and transfer from these fruits into water and unsweetened iced tea was examined. *Salmonella* survival on lemon flavedo is significantly higher ($P < 0.05$) after 24 h with storage at room temperature (2.45 log CFU/slice) than with storage on ice and refrigerated (below detection limit 0.95 log CFU/slice); the same is true for albedo at room temperature (1.43 log CFU/slice) compared with on ice and refrigeration (below detection limit 0.95 log CFU/slice) after 24 h. *Salmonella* populations survive poorly on lemon flesh; no significant difference exists between ice and room temperature storage, as populations remain below the detection limit (0.95 log CFU/slice) for the majority of the time points. Lime flesh supports *Salmonella* survival significantly better than lemon flesh at all time points. *Salmonella* inoculated onto lime flesh or albedo and held at room temperature or on ice does not decrease over 24 h. Populations on limes at room temperature have the greatest survival. The addition of flavedo or albedo-inoculated limes to chilled water results in the greatest *Salmonella* transfer into the beverage.

INTRODUCTION

It is a common food service practice to add lemon (*Citrus limon*) or lime (*Citrus aurentifolia*) slices to beverages *(23)*. The fruit is typically prepared at the beginning of the day and held for use, either at room temperature or on ice, throughout the remainder of the day. This task is performed by kitchen staff, servers, or bartenders who, if not adhering to proper handwashing protocols, may contaminate garnishes during beverage handling *(5, 7, 10)*.

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Citrus fruit are comprised of two distinct tissue regions; the flesh and the pericarp. The flesh, or core, of the fruit consists of locules in which seeds and juice sacs are located. The pericarp is further divided into two distinct sections: the exocarp, or flavedo, and the mesocarp, or albedo. The flavedo is the outermost, colored layer that includes oil glands (that produce oils, waxes, terpenes, and sesquiterpenes) and an outer, waxy layer. The albedo is the inner, white tissue consisting of parenchymatous cells and large air spaces *(21)*. The likelihood of *Salmonella* survival on sliced citrus may vary significantly depending on the region of inoculation. Oranges, a citrus variety that has a higher pH (ca. 3.8 *(33)*), are able to support *Salmonella* survival and have previously been identified as vehicles for salmonellosis outbreaks caused by consumption of contaminated juice *(19)*. A variety of viable bacteria genera, including *Acinetobacter, Bacillus, Corynebacterium, Enterobacter, Enterococcus, Escherichia, Klebsiella, Micrococcus, Proteus, Pseudomonas, Staphylococcus,* and *Streptococcus*, as well as fungal *Candida* spp., have been recovered from the flavedo and flesh of lemon slices (pH 2.3; *(23)*). The ability of *Salmonella* to survive over time on lemon or lime slices has not yet been verified, nor has potential transfer of *Salmonella* from contaminated garnish to beverage.

Lemons are frequently touted for their antimicrobial properties, which include a low pH (around 2.3; *(12)*) and the presence of essential oil compounds, including the terpene limonene *(11)*. However, essential oils are located predominantly within the flavedo, or outer peel, of the fruit *(9, 15, 42)*, which has a relatively neutral pH, while the flesh contains high levels of acid but low levels of limonene *(28)*. Therefore, there is likely little additive antimicrobial effect of these two components if pathogenic contamination occurs on any portion of the fruit surface. Many *Salmonella* serovars also have robust acid stress responses that enable them to tolerate a low pH environment for short periods of time *(22, 26)*.

No outbreaks have been linked to citrus used as a garnish for tea. However, two *Salmonella* outbreaks have been reported in teas: *S.* Enteritidis and Seftenberg in tea brewed from fennel, aniseed, and caraway have caused outbreaks in Serbia *(18)*, and *S.* Agona was traced back to a similar product imported from Turkey in Germany *(35)*. Wine *(26)* and beer *(27)* do not support the survival of *Salmonella*, likely because of the presence of compounds such as phenolics and/or hops, and ethanol. Low-acid beverages such as teas, colas and juices are not bactericidal against *Salmonella*, which was able to survive a pH of 2.7 when inoculated into cola for 5 minutes, indicating that the pH was not sufficient to inhibit the pathogen over short holding times *(26)*. The pH of black tea can range from 3 to 5 *(36)*, a distribution that includes the potential growth range for *Salmonella (8)*. *Salmonella* is also capable of long-term survival in water with temperatures ranging from 4 to 28°C *(25, 40)*. Additionally, tea leaves themselves may be contaminated by other organisms that may not be killed if the tea is improperly brewed; the coliforms *Kelbsiella* and *Enterobacter* can contaminate tea leaves *(7, 37)*, and even survive in commercially produced iced tea at levels in excess of 1,100 MPN/ml *(44)*.

The objectives of this study were to determine the fate of *Salmonella* when inoculated onto lemon or lime slices, which were then stored at room temperature for 24 h or stored on ice for 8 h and then transferred to refrigerated storage for 16 h (24 h storage total), and to determine the transfer of *Salmonella* from inoculated lemon or lime slices into a glass of chilled water or unsweetened iced tea.

MATERIALS AND METHODS

Beverage preparation

The two beverages used in this study were chilled tap water and iced tea. The tap water was obtained from the laboratory in Lake Alfred, FL and chilled to 4°C prior to use. The unsweetened iced tea (pH 6.8) was purchased from a local grocer (Winter Haven, FL, USA) and stored in the refrigerator for up to three days prior to experimentation.

Fruit preparation

The lemons and limes used in this study were purchased from a local grocery retailer (Winter Haven, FL, USA). Fruits were rinsed with tap water and dried prior to cutting. Cutting was done immediately prior to experimentation. Fruits were cut using a sterilized knife and cutting board. Each fruit was cut into eight equal sections weighing approximately 10 g each.

Selection of *Salmonella enterica* subsp. *enterica* serovars

A cocktail of five *Salmonella enterica* subsp *enterica* serovars isolated from orange juice outbreaks were used. *Salmonella* serovars and their sources were: *Salmonella* Gaminara (CDC 0662), *Salmonella* Rubislaw (F2833), *Salmonella* Typhimurium (ATCC 14028), *Salmonella* Hartford (CDC H0778), and *Salmonella* Meunchen (LJH 0592). All serovars were adapted to grow in the presence of 50 µg/ml nalidixic acid (NA; Sigma Aldrich, St. Louis, MO, USA) through the use of a stepwise exposure *(34)*.

Culture preparation

Prior to each experimental replication, the frozen culture was streaked onto tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD, USA) containing 50 µg/ml of NA (TSAN) and incubated at 35 ± 2°C for 24 ± 1 h. All *Salmonella* serovars were transferred to 10 ml of Trypic Soy Broth (TBS; Difco, Becton Dickinson, Sparks, MD, USA) with 50 µg/ml of NA (TSBN) and incubated at 35 ± 2°C for 24 ± 1 h; *Salmonella* serovars were transferred to fresh TSBN and incubated again at 35 ± 2°C for 24 ± 1 h. Cells were harvested by centrifugation at 3000 × g for 10 min. The cells were washed twice by removing the supernatant and suspending the cells in 10 ml of 0.1% peptone water

(Difco, Becton Dickinson). The cocktail was prepared by adding equal volumes of each serovar. Serial dilutions in 0.1% peptone water of the cocktail were performed to obtain a final cocktail concentration of 6 log CFU/ml. Inocula were enumerated on TSAN and used immediately to inoculate lemon and lime slices.

Inoculation

Lemon and lime slices at room temperature (24 ± 1°C) were inoculated with 20 µl of inoculum, distributed in 4–6 drops on the flavedo (colored exterior peel), albedo (white interior peel) or flesh and allowed to dry for 1 h. Fruit slices were placed into sterile 7 × 12 in. stomacher bags (WhirlPak; Nasco, Modesto, CA, USA). Two fruit slices were inoculated for each set of variables and sampling time.

Sliced fruit storage

Each fruit slice was stored individually in an open bag; bags were not closed in order to allow air movement that would better simulate real-world storage conditions. Fruit slices were stored on ice or at room temperature (24 ± 1°C). Fruit slices stored on ice were transferred to refrigerated storage, at 4°C, after 8 h on ice, to replicate storage conditions in a food service establishment. For all storage conditions, two fruit slices were taken at 0.25, 1, 2, 4, 6, 8, and 24 h for microbiological analysis; the experiment was repeated three times (n = 6).

Transfer to beverages

Lemon slices inoculated on the flavedo or albedo and lime slices inoculated on the flavedo, albedo, or flesh were allowed to dry 1 h at room temperature. Fruit slices were placed in 575 ml water or iced tea (starting at 4°C and being held at room temperature) in a glass beaker. Each beverage was stirred five times with a sterile concave-shaped metal rod (commonly referred to as a scoopula). Beverages were held 5–10 min prior to microbiological analysis.

Microbiological analysis

To enumerate *Salmonella* populations, 15 ml of Dey/Engley (DE; Sigma Aldrich) neutralizing broth was added to each fruit slice inside the stomacher bag. *Salmonella* were dislodged from fruit surfaces by use of a rub-shake-rub method for 30 s. Serial dilutions in 0.1% peptone water were spread plated in duplicate onto TSAN and bismuth sulfate agar (BSA; Difco, Becton Dickinson) with 50 µg/mL nalidixic acid (BSAN). Plates were incubated at 35 ± 2°C for 24 h (TSAN) and 48 h (BSAN). Following incubation, colonies were counted by hand, and *Salmonella* populations were converted to log CFU/slice of fruit.

Salmonella populations from beverages were determined by use of an MPN method. A three dilution by five tube MPN was performed, beginning with 100 ml, 10 ml, and 1 ml in equal volumes of double strength TSBN incubated at 35 ± 2°C for 18–24 h. Positive TSBN tubes were carried through the U.S. Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM) *Salmonella* enrichment method *(13)*. When *Salmonella* populations were enriched from the original TSBN tube, that tube was counted as positive for *Salmonella* in the MPN. Most probable numbers were determined using MPN tables supplied in the FDA BAM *(13)*, and *Salmonella* populations were reported as MPN/ml beverage.

Statistical analysis

All statistics were performed in JMP Pro 13.2 (SAS, Cary, NC, USA). An analysis of variance was used for the survival study treatment data, which were analyzed individually using a full-factorial design (mixed-model) with repeated measures. Student's *t*-test was used to test for significance of differences between sampling time mean values. Differences were considered significant at $P \leq 0.05$. Microbial transfer MPN results were replicated in triplicate, and percent transfer based on initial inoculum per individual replicate was reported. An analysis of variance using Student's *t*-test indicated significant differences among treatments ($P \leq 0.05$).

RESULTS

Fate of *Salmonella* on sliced lemons held at room temperature

Survival of *Salmonella* on sliced lemons held at room temperature is shown in *Fig. 1A*. Sliced lemons held at room temperature were inoculated with 5.0 ± 0.4 log CFU *Salmonella*/slice. Overall, all surfaces had statistically different levels of *Salmonella* survival; the flavedo supported the greatest survival of *Salmonella* (2.2 log CFU/slice), followed by the albedo (1.8 log CFU/slice), and the flesh at 1.2 log CFU/slice ($P \leq 0.05$). At the first sampling, populations on all three regions of the fruit experienced the same statistical reduction, ranging from 2.5 (flesh) to 3.0 log CFU/slice (flavedo); $P \leq 0.05$. By 2 h of storage at room temperature, *Salmonella* was no longer detectable on the flesh and remained undetectable for the entire 24 h sample period. The albedo surface had statistically the same reduction at 2 h as the flesh (1.3 log CFU/slice; $P \leq 0.05$), but the population recovered to around 1.9 log CFU/slice by 4 h and at 24 h was 1.4 log CFU/slice. The flavedo surface had statistically greater *Salmonella* survival at 2 h, with a population of 2.5 CFU/slice ($P \leq 0.05$); at 24 h the flavedo (2.4 log CFU/slice) had *Salmonella* at levels statistically the same as the albedo, but statistically higher than the flesh surface ($P \leq 0.05$).

Fate of *Salmonella* on sliced limes held at room temperature

Survival of *Salmonella* on sliced limes stored at room temperature is shown in *Fig. 1B*. Sliced limes held at room temperature were inoculated with 4.6 ± 0.7 log CFU/slice *Salmonella*. Overall, across all time points there was no statis-

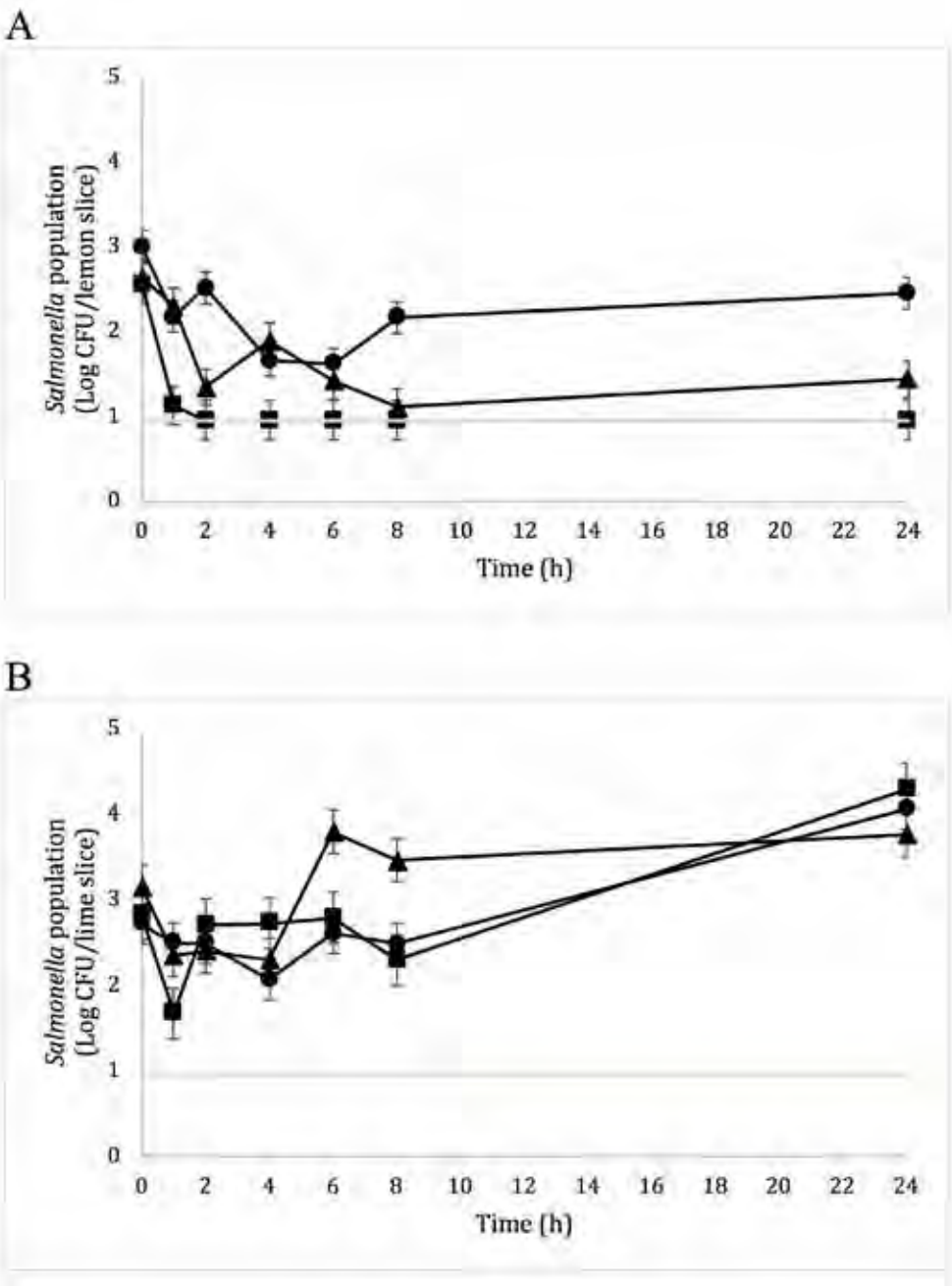

Figure 1. Survival of *Salmonella* inoculated onto flavedo (●), albedo (▲), or flesh (■) of sliced lemon (A) and lime (B) and stored at room temperature (24 ± 1°C). Results shown are those enumerated on TSAN. Solid gray line indicates lower limit of detection (0.95 log CFU/slice).

tical difference in populations among the three surfaces (P = 0.92). *Salmonella* populations inoculated onto all three surface declined to 1.7–2.5 log CFU/slice by 1 h. The *Salmonella* population on the flesh (4.3 log CFU/slice) was statistically greater at 24 h than at 0 h, and was statistically the same as on the albedo (3.8 log CFU/slice) and flavedo (4.1 log CFU/slice) at 24 h.

Fate of *Salmonella* on sliced lemons held on ice (8 h), then refrigerated (16 h)

Survival of *Salmonella* on sliced lemons stored on ice and then transferred to refrigeration is shown in *Fig. 2A*. Sliced lemons held on ice were inoculated to *Salmonella* populations of 3.0 ± 0.3 log CFU/slice. Across time points, *Salmonella* populations were not statistically different in relation to inoc-

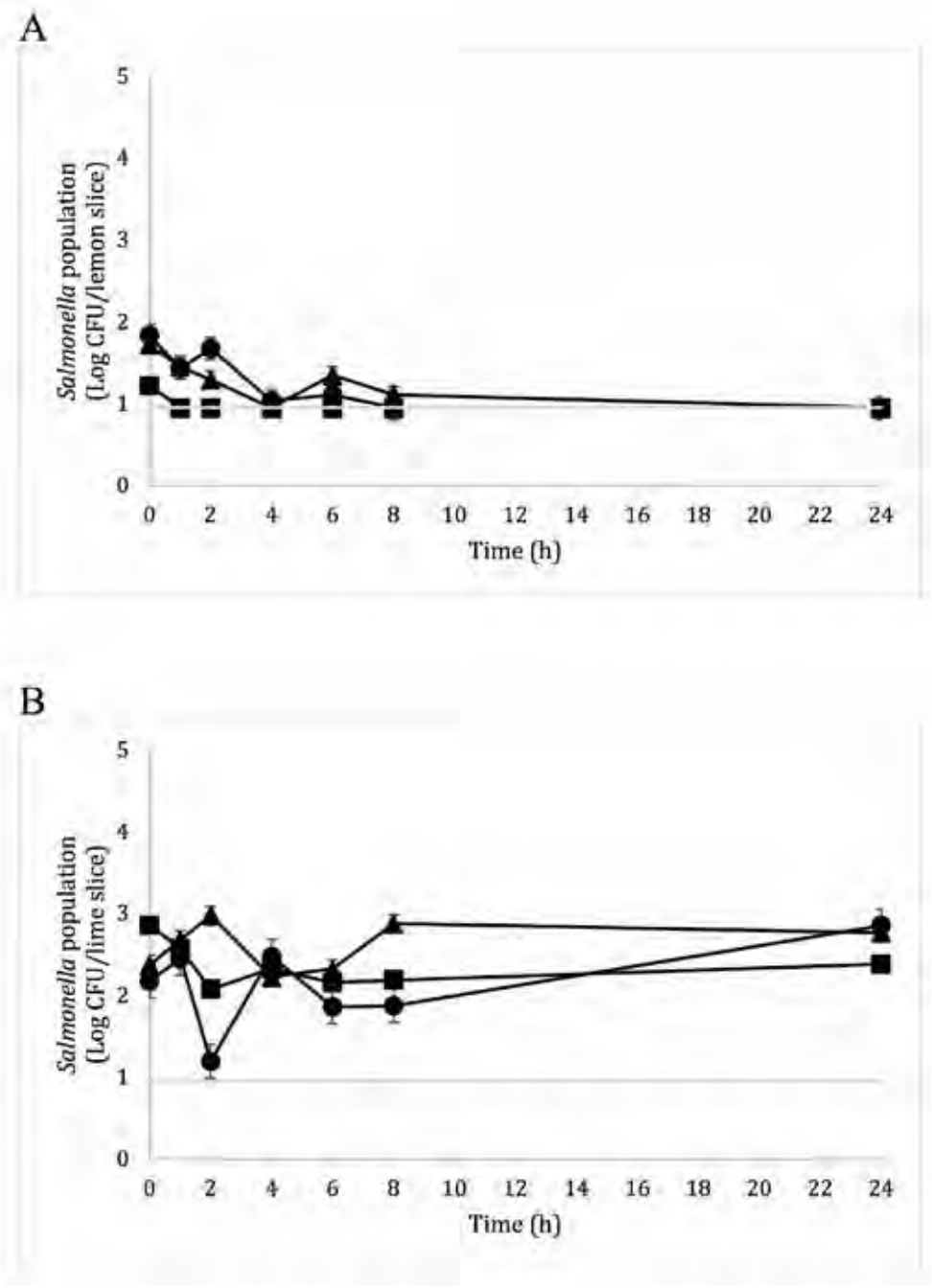

Figure 2. Survival of *Salmonella* inoculated onto flavedo (●), albedo (▲), or flesh (■) of sliced lemon (A) and lime (B) and stored on ice for 8 h, then transferred to refrigerated storage. Results shown are those enumerated on TSAN. Solid gray line indicates lower limit of detection (0.95 log CFU/slice).

ulated surface ($P = 0.06$). *Salmonella* on all three surfaces had similar reduction by the first sampling point (0 h), ranging from 1.2 to 1.8 log CFU/slice ($P \leq 0.05$). By 24 h, *Salmonella* was below the limit of detection (0.95 log CFU/slice) on all three surfaces.

Fate of *Salmonella* on sliced limes held on ice (8 h), then refrigerated (16 h)

Survival of *Salmonella* on sliced limes stored on ice and then transferred to refrigeration is shown in *Fig. 2B*. Sliced limes held on ice were inoculated to *Salmonella* populations

of 4.2 ± 0.6 log CFU/slice. *Salmonella* behaved very similarly on all three surfaces over the 24 h period ($P \leq 0.05$), with the exception of the 2-h time point, when the flavedo population was significantly reduced, to 1.2 log CFU/slice ($P \leq 0.05$). All three surfaces had the same reduction at 0 h, ranging from 2.2 to 2.9 log CFU/slice, at which point the populations remained stable through 24 h, during which time they ranged from 2.4 to 2.9 log CFU/ml.

Salmonella transfer from lemon and lime garnishes to beverages

Viable *Salmonella* transferred into water and tea from inoculated lemons and limes following a 5–10 min immersion. Percent transfer of the initial inoculum into the beverage was calculated using the following equation:

$$\text{percent transfer} = 100\left(\frac{\left(\frac{\text{MPN}}{\text{ml}}\ \text{measured in beverage} \times 575\text{ml}\right)}{\text{CFU inoculated onto slice}}\right)$$

Transfer from the flavedo and albedo of limes into water was >16 MPN/ml, resulting in a 9.5% rate of transfer from the initial lime inoculum (*Table 1*; $P \leq 0.05$). This transfer was statistically the same as for lemon flavedo in water (12.6%) and tea (8.6%), as well as lime flavedo (6.5%), albedo (9.5%) and flesh (5.7%) in tea. Transfer from lemon albedo into water (0.4%) and from lime flesh into water (0.9%) was statistically lower. However, transfer from lemon albedo into tea (0.3%) was statistically the same as transfer from lemon albedo and lime flesh into water.

DISCUSSION

Salmonella populations initially decreased on both the lemon and lime surfaces; *Salmonella* tended to fare better when inoculated onto the lime surface than when inoculated onto the lemon surface, both at room and refrigerated temperatures. The survival of *Salmonella* on lemons and limes is influenced by the interplay between intrinsic and extrinsic factors. Storage temperature *(45)*, fruit pH *(3)*, presence and concentration of essential oils and other antimicrobials related to plant defense *(43)*, natural microflora *(3, 29)*, and the ability of the pathogen to adapt to these stresses *(17)* are all integral to determining whether contamination of citrus garnishes poses a food safety threat during beverage service. *Salmonella* survival on flesh was generally lower regardless of fruit type or storage conditions, most likely because of the lower pH on the flesh than on flavedo and albedo surfaces. Slicing the fruit resulted in minimal flavedo damage and subsequent release of plant defense antimicrobials, resulting in a more hospitable environment than if the skin had been grated or zested.

The commonly reported pH of lemon and lime juices are 2.2–2.4 and 1.8–2.0, respectively *(12)*. The pH of fruit surfaces (flavedo, albedo, and flesh when juice vesicles are not broken) are assumed to be closer to neutral, as is true for oranges, in which the albedo has a pH of 6.0–6.5 while the juice has a pH of 3.8 *(33)*. *Salmonella* requires a minimum pH of 4.2 for growth *(13)*, which is well within the pH range experienced by microorganisms on the citrus surface. The sliced lemon surface supports the survival of an array of microorganisms; a variety of coliforms, Gram-positive bacteria, and yeasts have been isolated from the flesh or flavedo of lemon slices served in restaurants, although preparation and storage conditions were not reported *(23)*. Slicing of fruit ruptures tissue cells and releases nutrient-rich exudates for microorganism growth *(14)*; for example, salsa prepared with lime juice supported more growth of *Salmonella* compared with salsa made from the same recipe but without lime juice *(24)*. However, the same destruction of cells and juice sacs also increases the potential for contact between the microorganisms and the acidic components held within those cells or juice sacs. When 6.9 log CFU of an acid-tolerant *Salmonella* cocktail was inoculated onto 10 g of frozen lime juice concentrate (-23°C), no cells were recovered after 15 min or at any point thereafter during the entire 2-week trial period *(32)*. The current study utilized

TABLE 1. Quantitative percent transfer of *Salmonella* from inoculated lemons and limes to water or unsweetened tea. Within a beverage, means with the same superscript are not significantly different from each other ($P \leq 0.05$)

		Water		Tea	
		MPN/ml	% Transfer	MPN/ml	% Transfer
Lemons	Flavedo	10.2[a]	12.6	8.9[ab]	8.6
	Albedo	0.67[bc]	0.4	0.25[c]	0.3
Limes	Flavedo	>16[a]	>9.5	12.5[a]	6.5
	Albedo	>16[a]	>9.5	11.2[a]	9.5
	Flesh	1.7[bc]	0.9	11.5[a]	5.7

less acid tolerant strains of *Salmonella* under the assumption that cross-contamination in a food service environment is probably more likely from sources such as eggs, meat and poultry, products which themselves have relatively neutral pH *(12)*.

Storage temperature extrinsically controls microbial growth on cut fruit surfaces. The surface temperature of fruit in direct contact with ice is assumed to be close to 0°C. However, the fruit portion not in direct contact with ice is more likely to fluctuate in accordance with ambient environment temperature. The current study demonstrated that at room temperature, *Salmonella* on the flavedo of sliced lemons will survive at low concentrations for at least 24 h; a much more robust survival response occurs on the flavedo, albedo, or flesh of sliced limes held at room temperature. *Salmonella* survival on other cut fruit surfaces held at room temperature is not uncommon; cut mango, papaya, or dragon fruit held at room temperature are also capable of supporting *Salmonella* for at least 24 h *(38, 39)*. On the peeled orange surface (albedo), *Salmonella* populations can undergo a 2 log increase after 24 h at room temperature *(33)*. While ambient temperature is detrimental to *Salmonella* survival on low-moisture foods *(6, 41)* over prolonged periods of time when compared with survival at refrigerated temperatures, this is not the case on cut lemons and limes over the short time period of this experiment.

Similar studies have demonstrated that while decreases in the initial *Salmonella* inoculum do occur, the pathogen is still recoverable after 24 h refrigeration on cut mangos or papayas *(39)* and intact berries *(30)*, and after 2 weeks undergoes little decline on the peeled orange surface *(33)*. In no instance was *Salmonella* reduced to below the limits of detection on the aforementioned fruit surfaces. This is not unexpected, as refrigeration does not serve as a "kill step" against foodborne pathogens; its primary function is the reduction of bacterial growth on contaminated food. Refrigeration effectively controlled the growth of *Salmonella* on the lemon surface, reducing the population of the organism to below the limit of detection (0.95 log CFU/slice) within 4 h on all surfaces. On the chilled lime surface, *Salmonella* fared better, although the population was still reduced 100-fold after 24 h. Similarly, Dawson et al. *(10)* found that *Escherichia coli* populations decreased during refrigerated storage after 24 h, but viable bacteria were still present on the lemon surface; *E. coli* populations on lemons stored at room temperature (22°C) increased slightly by 24 h.

The flavedo of citrus fruits contains essential oils *(1)*, the constituents of which are bactericidal *(2, 16, 31)*. However, their efficacy against common juice spoilage organisms is limited; *Lactobacillus* and *Bacillus* species have shown to be particularly resistant *(4)*. The ability of *Salmonella* to survive on the flavedo of lemons and limes at ambient temperatures for 24 h indicates either that the efficacy of limonene as an antimicrobial is not sufficient to control pathogens or that limonene is not present in high enough concentrations on the undamaged flavedo surface to exert anti-*Salmonella* activity.

Development of acid tolerance is common in *Salmonella* *(22)*; the use of acid-adapted *Salmonella* under the same experimental conditions is a potential next experimental step to better understand the microbial risks involved in the storage of sliced lemons or limes and their subsequent use as beverage garnishes. Non-acid-tolerant *Salmonella* were used in this study to better mimic cross-contamination from a non-acidic source, which could conceivably be present in a food service environment. However, it would be relevant to perform this same analysis using acid-tolerant serovars to compare a worst case scenario situation.

Transfer rates from slices into water or tea were comparable to rates of transfer seen by Jung et al. *(20)* from the fruit surface onto gloved hands or to the edible portion of the fruit from the contaminated fruit surface. Jung et al. found that average transfer of *Salmonella* from the citrus peel to the edible fruit portion ranged from 0.16% to 5.41% and transfer from the peel to gloved hands ranged from 0.41% to 8.97%. While the current study differed in that passive transfer into liquid matrices was examined, rates of transfer were similar to those reported in the Jung et al. study, ranging from 6.5% to 12.6%.

CONCLUSIONS

Survival of *Salmonella* on lemon and lime garnishes, and transfer into chilled beverages, may occur, indicating that improperly handled and stored garnishes may be a potential vehicle for the transmission of foodborne illness. Storage of these garnishes on ice and under refrigeration decreased the growth of *Salmonella* populations on the fruit surface; the practice of keeping garnishes chilled will inhibit increases of *Salmonella* populations. Care must be taken to ensure that initial cross-contamination does not occur on these food items, as no step occurs to remove or kill pathogens once they contact the fruit surface.

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