Research Note

Fate of Salmonellae in Calcium-Supplemented Orange Juice at Refrigeration Temperature

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ABSTRACT

Recent outbreaks of salmonellosis associated with orange juice have raised interest concerning the survival and growth of *Salmonella* in juice supplemented with calcium. A study was done to determine the influence of various calcium supplements on the survival of salmonellae in orange juice held at 4°C for up to 32 days. Isolates of *Salmonella* Muenchen (inoculum 1), *Salmonella* isolates from humans and animals (inoculum 2), and *Salmonella* isolates from produce outbreaks (inoculum 3) were inoculated into pasteurized orange juices with pH values ranging from 3.96 to 4.19 and containing 350 mg of calcium per 240-ml serving (1.46 mg of calcium/ml). Populations of *Salmonella* declined rapidly in juice containing calcium lactate (CaL), with counts decreasing from 4.86 log_{10} CFU/ml to <1 log_{10} CFU/ml within 16 days, regardless of the *Salmonella* serotypes present in inoculum. Counts decreased from 4.89 log_{10} CFU/ml to <1 log_{10} CFU/ml in orange juice supplemented with CaL and tricalcium phosphate (TCP) within 30 days. These reductions were significantly (P < 0.05) higher than those of the control (no calcium added), in which *Salmonella* populations decreased 3.19 ± 0.20 log_{10} CFU/ml over 32 days. Populations in orange juice containing TCP or calcium citrate (CC) declined 1.34 ± 0.20 log_{10} CFU/ml and 1.96 ± 0.20 log_{10} CFU/ml, respectively, over 32 days. These counts were significantly higher than respective control counts in juice stored for 32 days. Populations of *Salmonella* of inoculum 3 inoculated into juice containing calcium citrate malate (CCM) were significantly higher than in the control. Higher numbers of cells in inoculum 3 also survived compared to numbers of cells of inocula 1 or 2 in juice supplemented with CCM. This study reveals that the form of calcium used to supplement orange juice influences the ability of salmonellae to survive.

Gastroenteritis is the most common form of salmonellosis caused by nontyphoid *Salmonella* and is characterized by nausea, vomiting, abdominal pain, headaches, elevated body temperature, and nonbloody diarrhea. Salmonellosis can be fatal in severe cases (14). In the summer of 1999, an outbreak of salmonellosis involving 298 confirmed cases and 1 death occurred in the western United States and Canada, in which unpasteurized orange juice was the vehicle of transmission (3). Potential sources of the pathogen included oranges, the processing-plant environment, and storage and transport facilities, although the actual source of contamination was not identified. Other outbreaks of salmonellosis associated with orange juice consumption have been recorded. In 1989, 67 cases of salmonellosis were reported in a New York hotel (2). The outbreak was linked to infected kitchen workers. In 1995, unpasteurized orange juice consumed at a Florida theme park was epidemiologically linked to 62 confirmed cases of *Salmonella* Hartford infection (4). A probable source of this contamination was amphibians that carried the pathogen into the juice-processing facility (12).

Salmonellosis associated with consumption of unpasteurized orange juice suggests that *Salmonella* can survive refrigerated, low-pH conditions at populations high enough and for periods long enough to cause illness. Warm oranges, surface inoculated with *Escherichia coli* O157:H7, can internalize the pathogen when placed in a cold inoculum (16). Growth of *E. coli* O157:H7 and *Salmonella* on surface-inoculated freshly peeled oranges has been reported (11). Compared to pasteurized fruit juices, unpasteurized juices present a public health risk because of the lack of a physical or chemical intervention designed to kill pathogenic microorganisms. Proper plant sanitation, as well as appropriate transport and storage temperatures, must be relied on to prevent the growth of pathogens in unpasteurized juice.

Consumption of fruit juices has increased in recent years. As fruit juice and soft drink consumption increase and dairy product consumption decreases in the United States (7), juice can be an effective delivery vehicle of calcium. These products also provide an alternative calcium source for lactose-intolerant individuals who have difficulty consuming dairy products. A 240-ml (8 oz) serving of calcium-supplemented orange juice provides 350 mg of calcium. Various forms of calcium, including a calcium lactate (CaL)-tricalcium phosphate (TCP) combination, calcium citrate (CC), and calcium citrate malate (CCM) are used to...
fortify juices. However, the effect of calcium supplements on the ecology of spoilage and pathogenic microorganisms in orange juice has not been reported.

The objectives of this study were to determine the effect of calcium supplementation of orange juice on survival of salmonellae during extended refrigerated storage and to determine if *Salmonella* Muenchen, a serotype implicated in an orange juice-associated outbreak, has unusual survival characteristics in orange juice compared to *Salmonella* serotypes isolated from sources other than orange juice.

**MATERIALS AND METHODS**

**Preparation of calcium supplements and orange juice.** Calcium supplements used to fortify commercial orange juice were evaluated. These consisted of CaL and CC, both supplied by Jungbunzlauer (Newtown, Mass.,) and calcium citrate malate (CCM), formulated in our laboratory according to a procedure described by Fox et al. The calcium carbonate (300 g) (J. T. Baker, Phillipsburg, N.J.) was slowly added to the solution and stirred for 3 h at 22°C. The solution was poured into a sterile, rectangular pan (53 by 33 cm), placed in a convection oven preheated to 80°C, and dried for 19 h. After cooling to 22°C, the CCM was removed from the pan and ground in a Wiley mill (Arthur H. Thomas Co., Philadelphia, Pa.) equipped with a 0.5-mm screen. The CCM powder was stored in sterile bottles at 22°C until used.

A commercially pasteurized orange juice made from concentrate was purchased at a local supermarket and stored at 4°C until used. This is referred to as the orange juice base.

**Strains used.** Three *Salmonella* preparations of inocula, each containing five strains, were used. Inoculum 1 was composed of five *Salmonella* Muenchen isolates provided by Dr. Ramesh Gauton at the State of Washington Department of Health (Seattle, Wash.). Inoculum 2 consisted of *Salmonella* serotypes isolated from human or animal sources (*Salmonella* Typhimurium, *Salmonella* Heidelberg, *Salmonella* Thompson, *Salmonella* Infantis, and *Salmonella* Enteritidis). Inoculum 3 contained *Salmonella* serotypes that were implicated in produce-associated outbreaks: *Salmonella* Gaminara (orange juice), *Salmonella* Hartford (orange juice), *Salmonella* Michigan (cantaloupe), *Salmonella* Baidon (tomato), and *Salmonella* Poona (cantaloupe).

A frozen stock culture of each strain was streaked on to tryptic soy agar (Becton Dickinson, Sparks, Md.) and incubated at 37°C for 24 h. Individual colonies of each strain were streaked on tryptic soy agar plates and incubated under the same conditions. Single colonies from each plate were then incubated into 10 ml of tryptic soy broth (Becton Dickinson). Strains were incubated overnight at 37°C with agitation (170 rpm) until cultures reached an optical density at 600 nm of 0.90 to 0.95. To prepare each inoculum, 1 ml of culture of each of five strains or serotypes was added to a 15-ml conical screw-cap tube (Becton Dickinson), creating a total of 5 ml of each inoculum in each of three tubes. Cells in each inoculum were then sedimented by centrifugation (2,700 × g for 20 min at 20°C). The supernatant fluid was decanted, and the cell pellet was resuspended in 5 ml of orange juice base at 4°C.

**Addition of calcium salts to orange juice.** Calcium salts were added to uninoculated orange juice base in amounts to give 350 mg of calcium per 240-ml (8 oz) serving, or 1.46 mg of calcium/ml. Amounts of each salt added separately to 100 ml of orange juice were as follows: 1.04 g CaL, 0.39 g TCP, 0.52 g CaL, and 0.19 g TCP, 0.69 g CC, or 0.69 g CCM. Each salt was added to a sterile 150-ml dilution bottle to which 99 ml of orange juice base was added. The mixture was shaken to dissolve the calcium salts. Most salts were readily solubilized, but some of the TCP remained in suspension. A control with no calcium salt added to the orange juice was also tested. Juice was stored at 4°C overnight until inoculation.

**Inoculation of orange juice.** Each of the three *Salmonella* inocula was diluted 100-fold in orange juice base immediately before inoculating into calcium-supplemented juice. Diluted suspension (1 ml) was added to 99 ml of each calcium-supplemented orange juice or control orange juice (no calcium added).

**Analysis of orange juice.** Inoculated juice was mixed and stored at 4°C for 4 h before shaking by hand for approximately 10 s and withdrawing samples to determine the population of *Salmonella*. Juice (1 ml) was serially diluted in 9 ml of sterile 0.1% peptone water and surface plated (0.1 ml, in duplicate) on bismuth sulfite agar (Becton Dickinson). Plates were incubated at 37°C for 24 h, and presumptive *Salmonella* colonies were enumerated. Uninoculated orange juice was also surface plated on bismuth sulfite agar. Orange juice was analyzed for populations of *Salmonella* 15 times during 32 days of storage at 4°C. Two replicate experiments were performed.

**Analysis of data.** Data were analyzed using multiple regression analysis with SAS software (SAS Institute, Cary, N.C.). Populations of *Salmonella* (log_{10} CFU/ml) were plotted against days of storage at 4°C. The slope of the survival curve for each inoculum in each orange juice was compared to that of the same inoculum added to the control juice (no calcium added). The slopes of survival curves of salmonellae in the three inocula in each calcium-supplemented orange juice were also compared to each other to determine if there were significant differences. All tests were performed at the 95% confidence interval.

**RESULTS**

**Storage study.** Initial (day 0) populations of *Salmonella* Muenchen in orange juice inoculated with inoculum 1 ranged from 4.76 to 4.86 log_{10} CFU/ml. Populations in juice inoculated with inocula 2 and 3 ranged from 4.78 to 4.81 log_{10} CFU/ml and 4.70 to 4.89 log_{10} CFU/ml, respectively, at day 0. Survival curves for *Salmonella* in orange juice containing various calcium salts at 4°C for 32 days are shown in Figure 1. Salmonellae were inactivated most rapidly in juice containing CaL, with an initial count of 4.83 log_{10} CFU/ml to <1 log_{10} CFU/ml within 16 days. *Salmonella* was inactivated less rapidly in juice containing CaL-TCP, decreasing from an initial population of 4.75 log_{10} CFU/ml to <1 log_{10} CFU/ml within 28 to 30 days. Rates of decrease in *Salmonella* populations in juices supplemented with CaL and CaL-TCP were significantly greater (P < 0.05) than in the control juice for all inocula. *Salmonella* counts decreased by 3.19 ± 0.20 log_{10} CFU/ml in control juice during the 32-day storage period, regardless of inoculum. Death of *Salmonella* was slowest in juice containing TCP, decreasing by 1.34 ± 0.20 log_{10} CFU/ml dur-
ing 32 days for all inocula, which was significantly less ($P \leq 0.05$) than that of the control. The population decline in juice supplemented with CC was $1.96 \pm 0.20 \log_{10} \text{CFU/ml}$ over 32 days, also significantly less than that of the control. The population of *Salmonella* in orange juice supplemented with CCM decreased $2.54 \pm 0.59 \log_{10} \text{CFU/ml}$ during 32 days. The rate of *Salmonella* inactivation in CCM-supplemented juice inoculated with inoculum 3 was significantly slower than that of the control; however, death of *Salmonella* in orange juice inoculated with inoculum 1

FIGURE 1. Survival of *Salmonella* in inoculum 1 (Salmonella Muenchen), inoculum 2 (human and animal isolates), and inoculum 3 (produce isolates) inoculated into orange juice supplemented with calcium and stored at 4°C for up to 32 days. Key: juice supplemented with calcium lactate (CaL) (closed diamond); calcium lactate-tricalcium phosphate (CaL-TCP) (closed triangle); calcium citrate malate (CCM) (open diamond); calcium citrate (CC) (open circle); tricalcium phosphate (TCP) (open triangle); and control (no calcium) (closed circle).
or 2 was not different from that of the control after 32 days of storage. Overall, there were minor differences in the survival of the *Salmonella* in the three inocula. The *P* values ranged from 0.26 to 0.69.

**DISCUSSION**

CaL was more inhibitory than other calcium salts to *Salmonella*. The CaL-TCP combination also rapidly reduced viability of *Salmonella* in orange juice compared to the control. Since TCP alone was not inhibitory to *Salmonella* in orange juice, it appears that the antimicrobial activity of the CaL-TCP combination was principally due to the lactate ion.

CaL has been applied to refrigerated fruit and fresh-cut fruit as a firming agent and to retain color (6, 9). However, its efficacy in controlling pathogens in fruit juices has not been reported. The low pH of fruit juices is amenable for the use of CaL as an antimicrobial. The *pK<sub>a</sub>* of lactic acid, the conjugate acid of CaL, is 3.86 (15), which is the pH at which the undissociated form of the salt (CH<sub>3</sub>CHOHCOOH) is in equilibrium with that of its dissociated form (CH<sub>3</sub>CHOHCOO<sup>-</sup>). Although the pH of the orange juice, 3.96, was higher than 3.86, it was low enough to allow a sufficiently high concentration of the nonpolar, undissociated ion to cross the cell membrane and acidify the interior of cell, disrupting the efflux of hydrogen ions out of the cell, leading to cell death. Many studies have revealed that sodium lactate salts have antimicrobial activity on meat products (1, 8, 10).

CaL is used commercially as a sole source of calcium to supplement orange juice. A combination of CaL-TCP is also used commercially. Because of confidentiality, the concentration of CaL-TCP in commercial juice is not obtainable. The ratio of the two calcium salts may not be the same as used in our study, where 50% of the calcium supplemented (175 mg/240-ml serving) was provided by CaL, and 50% was provided by TCP.

The enhanced survival of *Salmonella* in orange juice supplemented with TCP may have been influenced by a higher pH compared to that of the control (Table 1). While the addition of CaL to orange juice did not alter the pH, the addition of TCP increased the pH from 3.96 to 4.29, the largest increase effected by a calcium supplement. TCP, Ca<sub>10</sub>(OH)<sub>2</sub>(PO<sub>4</sub>)<sub>6</sub>-XH<sub>2</sub>O (X is undetermined), contributes hydroxide ions that increase the pH of the juice. Similarly, the addition of CC increased the pH of the orange juice to 4.19. The higher pH of the juice imposed less acid stress on the salmonellae. This corresponds to the observations of others, who reported that the length of time *Salmonella* survives in orange juice positively correlates with increased pH (13).

In summary, the addition of calcium salts to orange juice can significantly enhance or reduce the survival of *Salmonella*. Additional work is needed to fully assess the effect of CaL on sensory qualities of orange juice. Qualities such as sourness, staleness, and freshness of taste can be affected by calcium supplements. When used in fruit juices with different flavor characteristics, these effects may vary. CaL was the most effective of the supplements in inactivating *Salmonella*. The use of CaL to supplement commercial orange juice may simultaneously enhance nutritional value and inactivate *Salmonella* during storage.

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**REFERENCES**


**TABLE 1. Initial pH of calcium-supplemental orange juice**

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<td>Orange juice base</td>
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<tr>
<td>Calcium lactate (CaL)</td>
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