MANAN SHARMA
Survival of *Salmonella* in Orange Juice Fortified with Calcium
(Under the Direction of JINRU CHEN)

Outbreaks of salmonellosis in orange juice raised interest about the survival of *Salmonella* spp. in juice supplemented with calcium. Commercially calcium-fortified orange juice or juice that had been supplemented with calcium in the laboratory were inoculated with serotypes of *Salmonella* from orange juice, from humans and animals, and from produce-associated outbreaks, stored at 4$^{o}$C, and examined 15 times over 32 days. Juice containing calcium lactate (CaL) and CaL/tricalcium phosphate (TCP) showed more rapid reductions in counts in some *Salmonella* inocula in commercial and supplemented juice over controls (no calcium). Some *Salmonella* inocula counts in commercial and supplemented juices containing TCP and calcium citrate showed less rapid declines when compared with controls. *Salmonella* counts in juice containing calcium citrate malate showed a less rapid decrease in one instance when compared to controls. The form of calcium used to fortify orange juice impacts the survival of *Salmonella.*

INDEX WORDS: *Salmonella,* orange juice, calcium, fortification,
calcium lactate

# SURVIVAL OF *SALMONELLA* IN ORANGE JUICE FORTIFIED WITH CALCIUM

by

MANAN SHARMA

B. S., University of Florida, 1996

A Thesis Submitted to the Graduate Faculty
of The University of Georgia in Partial Fulfillment
of the
Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA
2000

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MANAN SHARMA

Approved:

Major Professor: Dr. Jinru Chen

Committee: Dr. Larry Beuchat
Dr. Michael Doyle
Dr. Mark Harrison

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
December 20000

# DEDICATION

To Mom, Pop, Dada ji, Dhai ji, Nani, and Bapu-Sahib

ACKNOWLEDGMENTS

Many people have supported me to throughout my education, and although the words written on this page cannot begin to thank them for their contributions to my life, I would like them to know how much I learned from all of them. I would like to thank the following people:

Dr. Jinru Chen, for her guidance, insight, creativity, patience, and encouragement.

Dr. Larry R. Beuchat for his guidance and support.

Dr. Mark Harrison and Dr. Michael Doyle for serving on my advisory committee and through this learning process.

The faculty and staff at CFSQE, especially Dr. Ying Mao, Dr. Mahbub Islam, Joy Adams, Dr. Chyer Kim, Andrea Burnett, Kim Hortz, Alan Scouten, Vijaya Mantripragada, Bob Flewellen.

My teachers, from elementary to graduate school, who always attempted to make learning and thinking a part of my life.

Jerry Davis, for his advice and guidance in statistical analysis

The students at CFSQE: Scott Burnett, Bill Weissinger, Peter Taormina, Steve Kenney, Sarah Holliday, Yong Soo Jung, and Radha Bheem-Reddy for their encouragement and support throughout the process.

My extended family, from Huntsville to Jaipur and points in between, for their love and encouragement.

My parents, whose love, support, humor and optimism have always been there for me, for making me understand the value of hard work and instilling in me the value of education.

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# INTRODUCTION AND LITERATURE REVIEW

***Introduction***

*Salmonella* spp. are facultatively anaerobic gram-negative rods in the *Enterobacteriaceae* family. The pathogen was first described in concurrent works of Salmon and Smith in 1885 as the causative agent of hog cholera, *Bacillus cholerae-sui*s (Tauxe, 1991). White proposed an antigenic classification scheme in 1926, later expanded by Kaufmann in 1941, presently known as the Kauffmann-White scheme (Tauxe, 1991). As of 1998, there were 2,435 serotypes identified by this method (Brenner and McWhorter-Murlin, 1998).

*Salmonella* spp. can be identified by several distinct biochemical characteristics. It is chemo-organo-trophic; it catabolizes D- glucose into acid and gas; it is oxidase negative and catalase positive (characteristic of facultative anaerobes); it is capable of growth on citrate as a sole carbon source; it does not utilize lactose or sucrose, and it does not hydrolyze urea (Andrews et al, 1992). It produces hydrogen sulfide and decarboxylates lysine and ornithine into alkaline by-products (D'Aoust, 1997).

Salmonellae have an optimum growth temperature of 37$^{o}$C. Serotypes have been known to grow at temperatures ranging from 4$^{o}$C to 54$^{o}$C (D'Aoust, 1991). The optimum pH range for growth is between 6.5 and 7.5, while the overall range spans from 4.5 to 9.5. It is commonly accepted that salmonellae do not grow in foods that have a water activity of less than 0.93. The presence of 3 to 4% NaCl also inhibits the growth of the organism, but this effect can be moderated with increasing growth temperature in the range of 10-30$^{o}$C (D'Aoust, 1997). The D-value, the amount of time at a certain temperature to kill 90% of the viable organisms, varies depending on the food medium (Jay, 1996).

***Classification***

For solely epidemiological classification, there are three groups of salmonellae. Group 1 are those that only affect humans - agents of typhoid and para-typhoid fever. Group 2 are referred to as host adapted serovars, those that live in humans or animals. This category contains many human pathogens that can be disseminated through food. Group 3 are unadapted serovars that have no host preference and are pathogenic for humans as well as other animals. Most *Salmonella* spp. foodborne pathogens fall into this category (Jay, 1996).

For taxonomic classification, there are two species in the genus *Salmonella*: *S. enterica*, which contains five subspecies (I, II, III, IV, and VI), and *S. bongori*, which was formerly referred to as subspecies V in *S. enterica*. There are 2,435 *Salmonella* serotypes in these two

species: 2,415 serotypes in *S. enterica*, 20 serotypes in *S. bognori*. These species and subspecies are differentiated on biochemical properties. Strains are serotyped on the basis of their somatic (O), capsular (Vi), and flagellar (H) antigens. Most human *Salmonella* isolates reside in subspecies I (*S. enterica* supsp. *enterica*). Most of these strains are isolated from humans or other warm-blooded mammals. For subspecies II, III, IV, and VI, most strains are isolates from cold-blooded animals or the environment, and rarely contain human isolates. *S. bongori* strains are usually isolated from the environment (Brenner and McWhorter-Murlin, 1998). New serotypes classified in *Salmonella* subspecies I are named after the geographical location where they are first isolated. New serotypes classified in other subspecies or species are not given names but rather identified by antigenic formula (Brenner and McWhorter-Murlin, 1998).

***Salmonellosis***

Approximately 150 of all *Salmonella* serovars have been associated with human illness (Doyle and Cliver, 1991). Non-typhoid salmonellae usually affect individuals 8 to 72 hours after exposure to the causative agent. Enteritis is the most common form of salmonellosis, and is characterized by nausea, vomiting, abdominal pain, headaches, elevated body temperature and diarrhea (Jay, 1996). Most affected by the disease are those who are immunocompromised: children, whose immune systems are not completely developed, and the elderly, who produce decreased amounts of stomach acid, reducing one of the body's protective barrier against foodborne pathogens. Those who suffer from medical conditions like AIDS or cancer are also at greater risk (Salyers and Whitt, 1994, Murray et al, 1994). Most cases resolve themselves within five days after the onset of symptoms (Murray et al, 1994). Fluid and electrolyte treatment is recommended for more severe cases (Murray et al, 1994). Antibiotic treatment is usually not recommended because it can prolong the carrier state of individuals shedding the organism in feces (D'Aoust, 1997). Antibiotics also eliminate the natural microflora of the gut that compete with *Salmonella,* possibly extending the duration of illness (Doyle and Cliver, 1991, Murray et al, 1994). Aseptic reactive arthritis and Reiter's syndrome and ankylosing spendylitis are all chronic conditions that can be incurred through exposure to salmonellae (D'Aoust, 1997)

The infectious dose of salmonellae is dependent on several factors. The susceptibility of hosts and the virulence of *Salmonella* serotypes differ greatly. Also, the food vehicle in which

the bacteria is transmitted can also play a role. High levels of fat, as in ice cream, chocolate or butter, protect the bacteria from hydrochloric acid in the stomach. This could make the infectious dose as low as $10^1$-$10^2$ cells (Doyle and Cliver, 1991). Others have stated that $10^6$-$10^9$ *Salmonella* cells are needed to cause gastroenteritis (Jay, 1996, Murray et al, 1994).

***Epidemiology***

The Centers for Disease Control and Prevention (CDC) estimate that non-typhoidal *Salmonella* is responsible for over 1.3 million cases of foodborne illnesses annually, or 9.7% of those caused by known foodborne pathogens (Mead et al, 1999). It is responsible for 15,608 hospitalizations (25.6% of total foodborne hospitalizations) and 553 deaths (30.6% of total foodborne deaths) of those incidents caused by identified foodborne pathogens (Mead et al, 1999). These are the highest levels for any bacterial foodborne pathogen. Tauxe (1991) estimated that overall patient related costs of salmonellosis cases range between $275 million and $1.1 billion per annum in the United States. There had been a 15% decrease in the occurrence of *Salmonella*, dropping from 14.5 / 100,000 in 1996 to 13.6 / 100,000 in 1997 to 12.4 / 100,000 in 1998 (CDC, 1999). However, 1999 estimates show an increase to close to 1996 levels of 14.8/ 100,000 (CDC, 2000). The decrease in the number of *Salmonella* cases from 1996 to 1998 is widely thought to be due to implementation of Hazard Analysis of Critical Control Points (HACCP) programs in poultry and beef processing operations, along with new microbiological standards for egg quality (CDC, 1999). Even in 1999, levels of two of the serotypes routinely isolated, *S.* Enteritidis and *S.* Typhimurium, were relatively constant or declined (CDC, 2000). In 1999, there were salmonellosis outbreaks involving *S.* Muenchen (unpasteurized orange juice), *S.* Newport (mangos), and *S.* Mbandaka (raw sprouts). Reported incidence of both serotypes *S.* Muenchen and *S.* Newport increased 348% and 79%, respectively, from 1998 to 1999 (CDC, 2000).

This increase represents the ongoing shift in the current food safety paradigm to evaluate fruits and vegetables as vehicles of foodborne illness and to consider them just as important contributors to foodborne outbreaks as food animal products. The changing dietary habits of Americans continue to drive the consumption of more fruits and vegetables. DeRoever (1998) reported a 27% increase in the consumption of fresh produce from 1970 to 1993. This increased nutritional consciousness, combined with ever improving epidemiological investigative tools, lead one to believe that an increasing number of foodborne outbreaks will be ascribed to fruits

and vegetables. According to Tauxe (1991), 59 % of the *Salmonella* outbreaks that occurred between 1983-1987 were of an unknown food vehicle. This presents a daunting challenge of lowering the foodborne pathogen level from foods that traditionally do not undergo a "kill step" to neutralize pathogens. It is further magnified by the 38% of fruits and 12% of vegetables were imported last year, according to the Food and Drug Administration (Satcher, 2000). This is not to propagate the longstanding assumption that sanitary standards in developing countries are inferior; rather, it is to illustrate the public health challenges that await produce safety in the 21st century.

***Reservoirs and Survival***

Salmonellae, naturally present in animal and human gastrointestinal tracts, are excreted into the environment through feces. Poultry and beef animals raised in intensive husbandry settings contribute to spread of the organism among flocks and herds (D'Aoust, 1994). Also, exposure of food animals to birds, reptiles, rodents and insects, all of which can act as carriers, contributes to incidence of *Salmonella* (Beuchat, 1996). These animals can transfer salmonellae from raw sewage or manure to domestic animals or crops. Other considerations are parental transmission from mother to offspring and the contamination of animal feedstuffs.

There are many sources of potential produce contamination throughout the food chain. Beuchat (1996) elegantly describes such an interaction from pre-harvest, post-harvest and distribution. Pastures or farms where livestock animals grazed have a larger chance of being contaminated with pathogens (Tauxe, 1997). Flooding can be problematic when animal wastes or sewage treatment plants contribute effluents and transfer pathogens to agricultural land (Brackett, 1999). Some microorganisms can survive for months or years in croplands (Watkins and Sleath, 1981). The use of improperly treated sewage or manure in place of chemical fertilizers can also add the significant risk of pathogen contamination (Beuchat and Ryu, 1997). As organically grown produce continues to gain prominence, this may become more of a factor in produce safety. The use of contaminated water in surface or overhead irrigation facilitates the rapid dissemination of pathogens through some lands (Brackett, 1999). Fecal coliform counts of over 1,000 organisms/100 ml in irrigation water indicated the presence of *Salmonella* in 96.4 % samples. In contrast, when the fecal coliform count was between 1-1000 / 100 ml, *Salmonella* was only detected in 53.5 % of the samples (Geldreich and Bordner, 1971).

Survival of *Salmonella* in soil is related to inoculum, soil type, moisture retention, pH, microbial antagonists and nutrient availability (Geldreich and Bordner, 1971).

During harvesting, worker hygiene is of utmost importance. Salmonellae can spread through the fecal-oral route from workers with poor sanitary practices. During post-harvest food processing, equipment, containers, sanitation, hygiene, rinse water, ice quality, and proper temperature maintenance all contribute to overall produce safety (Brackett, 1999, Beuchat and Ryu, 1997).

The growth, survival and inactivation of bacteria in fresh fruit and vegetables depend on four factors: (1) the innate characteristics of the bacteria; (2) the physiological condition of the plant tissue and resistance to microbial activities; (3) the environment surrounding the plant tissue, such as pH, water activity, etc; and (4) the effects of processing not only on the bacteria but also on the plant tissue itself (DeRoever, 1998). Pao et al. (1998) demonstrated that *Salmonella* can not only survive but actually grow on peeled oranges; similar results were fount on fresh cut tomatoes (Zhuang et al, 1995). The microbial ecology of the specific vegetable or fruit may also play a role in survival of *Salmonella*. *Salmonella* contamination was twice as likely to occur in fruits and vegetables that were infected with the bacterial soft rot causative agents, *Erwinia carotovora* and *Pseudomonas fluorescens*, than those that were not (Wells and Butterfield, 1997). The pectolytic breakdown of the tissue leads to softening and liquefaction internally, allowing more favorable conditions for bacterial survival (Wells and Butterfield, 1997). The actual fruit may also provide growth niches, like the stem scar area on oranges and apples, where bacteria may be protected from the environmental stresses or physical or chemical sanitizing treatments (Pao and Davis, 1999).

***Citrus and Pathogens***

The external environment of the fruit can impact how *Salmonella* are taken up on or into fruit. Merker et al (1999) reported that warm oranges (21$^{o}$C) immersed in cold dye (4$^{o}$C) took up dye at a rate of 3.3 % . It is thought that the internal gases of the fruit contract, allowing fluid to be drawn into the fruit. This finding was supported by Walderhaug et al (1999), who reported that 3.6 % of oranges, surface inoculated with *E. coli* O157:H7, internalized the pathogen through the same mechanism as the dye. Tissue breaks, punctures, wounds, cuts, splits, insect penetration and thorns can also allow the penetration of bacteria from the surface to the inside of

the fruit (Anonymous, 1999). Fecal contamination in an orchard or contaminated water used in washing or removing field heat can also lead to such conditions.

Orange packinghouse procedures seem to cause a reduction in the surface microbial load of the fruit. In a study by Pao and Brown (1998), unwashed fruit entering seven packinghouses had total microbial loads of 4.0 $\log_{10}$ CFU (colony forming units)/$cm^2$. After undergoing dumping, washing, brushing, rinsing, water elimination, wax application and drying, and final hand packing, this microbial load decreased to 2.1 $\log_{10}$ CFU/$cm^2$ on the fruit surface. Generic *E. coli* was detected on the oranges before entering the packinghouse, but none was found after packing. Other fecal coliforms isolated from these fruit before waxing include *Klebsiella pneumoniae*, *Enterobacter spp.*, and *Citrobacter freundii*. However, no *Salmonella* was recovered at any stage during the process. Packinghouse procedures also lowered the level of yeasts, molds and aciduric organisms.

Growth of *E. coli* O157:H7 and *Salmonella* on surface inoculated freshly peeled orange pieces occurred at 24$^o$C (Pao et al,1998). These authors also reported that *E. coli* O157:H7 and *Salmonella* did not grow at refrigeration temperatures of 4 or 8$^o$C. A one $\log_{10}$ CFU/ML reduction was observed in *Salmonella* over the course of the shelf life of the peeled oranges at 4 and 8$^o$C, respectively. No corresponding reduction was observed with *E. coli* O157:H7. The pH of the surface of the peeled fruit (6.0-6.5) was much higher than that of the juice inside (3.8), indicating that refrigeration is a crucial element in preventing the growth of pathogens on the surface at this pH.

Some physical treatments have been evaluated to reduce pathogen levels on the surface of oranges. Immersion of oranges inoculated with *E. coli* in 70$^o$C water for 2 min and 80$^o$C water for 1 min water achieved a 5 $\log_{10}$ CFU/ $cm^2$ reduction of *E. coli* in non-stem scar areas (Pao and Davis, 1999). This treatment provided no discernable difference in flavor characteristics between juice that was made from heat-treated oranges and non-heat treated oranges. In the same study, a 5 $\log_{10}$ CFU/$cm^2$ reduction was achieved at the stem scar area at 70$^o$C for 4 min and 80$^o$C for 2 min, respectively, but there were significant flavor differences between the heat-treated and non-heat-treated juice.

Chemical treatments have proven less effective than physical ones in reducing bacterial levels on orange surfaces. Immersion of oranges in 100 parts per million (ppm) chlorine dioxide for 8 min at 30$^o$C resulted in a 3.1 $\log_{10}$ CFU/$cm^2$ reduction of *E. coli* in non-stem scar areas, whereas only an 1 $\log_{10}$ reduction was observed in stem scar areas (Pao and Davis, 1999).

Deionized water reduced counts by 2.0 and 0.6 $\log_{10}$ CFU/cm$^2$, respectively. Various other sanitizers, 100 ppm chlorine, 200 ppm acid anionic sanitizer, 80 ppm peroxyacetic acid, and 2% trisodium phosphate, did not reduce *E. coli* by more than the chlorine dioixde levels. Chemical sanitizers may be limited by their inability to penetrate the orange surface (Pao and Davis, 1999).

Alkaline sanitizers have proven more effective in decontaminating the surfaces of oranges. When compared with water, acid anionic cleaner (phosphoric acid, dodecylbenzene, sulfonic acid and isopropyl alcohol), 2 % sodium orthophenylphenate (SOPP) in detergent at a pH of 11.8 proved more effective in reducing *E. coli* counts on inoculated oranges (Pao et al, 2000). SOPP reduced counts by 2.9 to 3.5 log CFU/cm$^2$, regardless of any prewetting treatment used. The high alkalinity damages the structure of Gram negative cell walls (Mendonca et al, 1994). When compared with a sodium hydroxide (NaOH) solution and an alkaline cleaner (sodium and potassium hydroxide with other surfactants) adjusted to a pH of 11.8, there were no statistically significant differences in reduction of counts. However, SOPP provided the largest reduction in *E. coli* counts (Pao et al, 2000). This indicates that although the alkalinity provides most of the sanitizing effect, there is some contribution by the o-phenol group in the SOPP. In a previous study, fruit that was washed in a SOPP solution did not provide a reduction in microbial levels when compared to water (Pao and Brown, 1998). This suggests the volume and application time of the solution play a role in the effectiveness of SOPP.

High pH citrus waxes have also been shown to significantly reduce *E. coli* counts on the surfaces of oranges (Pao et al, 1999). Waxes are used for a variety of reasons: to reduce water vapor loss, enhance surface shine, or even to transmit an antimicrobial agent. Waxing oranges involves the application of a high pH wax (>8.0) followed by drying at 50-55$^o$C for 2-3 minutes. Spraying is the most common form of application, but dips, drips and foams are used on a lesser scale. Pao et al. (1999) reported that a 5 $\log_{10}$ CFU/cm$^2$ reduction of *E. coli* was achieved on an inoculated glass slide that was dipped in shellac wax for 4 minutes at 50$^o$C at a pH of 10. Using the same conditions on oranges, a reduction of approximately 4.7 $\log s_{10}$ were observed on mid-section areas, while a 1 $\log_{10}$ CFU/cm$^2$ reduction was observed on the stem scar area. Similar results were also seen at a pH of 11 at the same temperature for 2 minutes (Pao et al, 1999). Waxing also reduced the fecal coliform counts from 35.2 MPN / cm$^2$ to 1.4 MPN / cm$^2$ (Pao and Brown, 1998).

### *Orange Juice and* Salmonella

In a study performed by Parish et al. (1997), several serotypes of *Salmonella* (Hartford, Gaminara, Rubislaw, and Typhmurium) were grown in orange serum adjusted to pH 5.0 (for acidic adaptation) before inoculation into orange juice. Salmonellae inoculated in orange juice ($10^6$ CFU/ml) survived for 27 days at pH 3.5, 46 days at pH 3.8, 60 days at pH 4.1, and 73 days at pH 4.4. Survival times increased with increasing pH. Death rates were inversely related to pH, with juice at pH 3.5 showing the most rapid rate (Parish et al, 1997). There were no differences in serovar behavior in juices at pH 4.1 or 4.4; only two ( *S.* Rubislaw and *S.* Hartford) showed differences between pH 3.8 and 4.1 at 4$^o$C. This study represents an extreme case, where acid adapted cells could infiltrate a juice making process. The high pH of the peel (6.0-6.5) make it unlikely for *Salmonella* to encounter this type of environmental stress. Poor sanitation in a processing facility could potentially create this type of environment in an orange juice processing facility.

In a study performed with *E. coli* O157:H7 strains containing green fluorescent protein, the organism survived for 24 days in orange juice with a minimal decrease in cell population ( 1.48 and 1.84 $\log_{10}$ CFU/ml, respectively), starting from approximately 4 $\log_{10}$ CFU/ml (Fratamico et al, 1997). However, when these same strains were inoculated into apple cider, there were no detectable organisms after 24 days (Fratamico et al, 1997). *E. coli* O157: H7 survived the acidic conditions of orange juice better than apple cider – whether or not this has any implications for *Salmonella* survival has yet to be determined.

### *Orange Juice Outbreaks*

Over the last century there have been several orange juice outbreaks that have caused gastrointestinal illness associated with either viral or microbial agents. Table 1 summarizes these outbreaks. Food handlers who were either asymptomatic carriers or infected with the causative agent caused the 1944, 1962, and 1989 outbreaks. The 1944 outbreak occurred in a Cleveland hotel from an infected worker preparing orange juice (Duncan et al, 1946).
The 1962 outbreak was purportedly caused by an asymptomatic hospital worker preparing orange juice from frozen concentrate, whose spouse had symptoms resembling that of Hepatitis A (Eisenstein, 1963). The 1989 outbreak, the largest foodborne *S. Typhi* outbreak in nearly a decade, was thought to have originated from an infected kitchen worker (Birkhead et al, 1992).

TABLE 1. Outbreaks of Bacterial or Viral Origin in Orange Juice

| Year/Location | Causative Agent | Cases of Illness/ Death |
|---|---|---|
| 1944/Ohio | *Salmonella* Typhi | 18 / 1 |
| 1962/Missouri | Hepatitis A | 24/0 |
| 1989/New York | *S.* Typhi | 67/0 |
| 1992/Faridpur, India | Enterotoxigenic *E. coli* (ETEC) | Undetermined |
| 1995/Florida | *S.* Hartford<br>*S.* Gaminara | 62/0 |
| 1999/U.S. Pacific Northwest and Canada | *S.* Muenchen | 298/1 |

The orange juice was reconstituted from frozen concentrate under poor sanitary food preparation conditions. The preparation area was near a bathroom not equipped with soap or towels. Also, the concentrate was reconstituted in a large vessel, then serving containers were dipped into the vessel. This provided ample opportunity for worker hand contact with the juice. The estimated medical costs of this outbreak were $170,430 for all ill persons (Birkhead et al, 1992).

The outbreaks described above were caused by infected food handlers. However, over the last decade, a trend has emerged of the pathogen infiltrating the juice at the processing site. Also, as food consumption patterns have changed, ready to serve orange juice has become more popular, exposing more people to a potential juice-borne outbreak. The 1992 outbreak of enterotoxigenic *E. coli* (ETEC) in India was traced to four roadside orange juice stands that sold fresh squeezed unpasteurized juice. The location of one stand was near a garbage heap. All stands were exposed to high levels of dust (Singh et al, 1995). Therefore, it is difficult to state the origin of the ETEC in this case – whether it was an external factor or something intrinsic within the orange juice.

In the 1995 outbreak at a Florida theme park, contamination occurred during the processing of unpasteurized orange juice. Although 62 patients were confirmed with salmonellosis, the CDC estimated that 630 to 6,300 people were affected (Parish, 1997). One

patient had both *S.* Hartford and *S.* Gaminara, while the rest were affected by the Hartford serovar. Salmonellosis due to this serovar is rare, making up only 0.2 % of all salmonellosis cases on an annual basis (Cook et al, 1998). The majority of the orange juice at the theme park was produced at one processing facility. Extensive sampling of incoming oranges, bottled juice, equipment surface areas, and environmental areas outside of the plant (including toads, frogs, frog feces and environmental water) was performed (Parish, 1998). Samples were tested for fecal and total coliforms, *E. coli,* and *Salmonella. S.* Newport and *S.* Hartford were isolated from toads, while *S.* Newport were isolated from frogs. *S.* Rubislaw was isolated from bottled juice. One *Salmonella* serovar was also isolated from the unwashed surface of an orange. The Hartford strain isolated from patients of the outbreak was not found at the plant. However, the presence of the other *Salmonella* serovars indicates opportunity for contamination of the juice (Parish, 1998). Orange handling and washing was performed in an unenclosed structure, allowing contact between the amphibians, oranges and orange juice. Also, fecal coliform counts on juice contact surfaces were detected, suggesting that sanitary conditions during juice processing were poor (Parish, 1998). The poor sanitary conditions, combined with the inadequate security measures that allowed the environmental contamination of the juice, contributed to this outbreak.

The 1999 *S.* Muenchen outbreak also implicated unpasteurized ready to serve orange juice, resulting in one death and 298 confirmed illnesses. The distribution of this product through several western U.S. states and two Canadian provinces made it geographically more widespread than the theme park outbreak. The source of the serotype Muenchen remains unclear, although oranges, plant equipment surfaces, or transport equipment could have been potential sources (CDC, 1999)

The significance of these outbreaks shows that *Salmonella* can survive the low pH conditions of orange juice for a significant duration of time if there is no physical or chemcial intervention. Although it has been observed that salmonellae can survive under low pH conditions, it had been thought that the pH of orange juice would be sufficient enough to kill the pathogen to diminish its threat as a public safety concern. This assumption seems less valid in light of previous outbreaks.

Calcium may play a direct role in bacterial growth and survival. The lipopolysaccharide (LPS) layer of *S.* Typhimurium has high affinity binding sites for calcium, possibly indicating that calcium ions help stabilize this layer, promoting structural integrity (Smith, 1995). The

presence of calcium ions helped increase pressure resistance of *E. coli* cells (Hauben et al, 1998). The addition of EDTA, a calcium chelator, has been shown to induce premature cell division. The element is also though to play a role in DNA-protein binding (Smith, 1995).

***Calcium and Sodium Lactate Salts***

The addition of a GRAS (Generally Recognized As Safe) chemical that could help decrease pathogen loads in unpasteurized juices is a possibility. Orange juice processors add various calcium salts to fortify pasteurized product for nutritional purposes. Calcium lactate (CaL) could act similarly to sodium lactate (NaL), which has proven to be an effective antimicrobial against a number of pathogens in various food products (Maca et al, 1999, Banks et al, 1998, Eckert et al, 1997). Calcium, potassium and sodium lactates are considered GRAS and approved for general purpose usage by the FDA (Doores, 1993).

Calcium lactate is used for a variety of purposes: to prevent ropiness and coliform growth in bread, as a firming agent, a coloration inhibitor in apple slices, and to improve the quality of dried milk powder (Shelef, 1994). Lactate works as an antimicrobial by two possible mechanisms: 1) by lowering the water activity in the media, or 2) its weak lipophilic acid (the protonated, undissociated form) structure allows it to pass through the cell membrane, dissociate within the cell, lowering the internal pH of the cell, overwhelming the hydrogen ion transport mechanism of the bacterial cell and cause cell death (Shelef, 1994). This effect is more pronounced when the external pH is lower than the internal pH of the bacterial cell (Shelef, 1994). The $pK_a$ of lactic acid, the conjugate acid of the salt, is 3.86. Some work has shown that sodium lactate is more inhibitory to *S.* Typhimurium than sodium chloride (NaCl) at the same water activity (DeWit and Rombouts, 1989). It was also observed that sub-optimum growth temperature enhanced the effect of the sodium lactate. These authors postulated that lactate could have an increased permeability into the cell (an active transport mechanism) over NaCl, which might allow for the possibility that lactate salts are effective at pH values above their $pK_a$ (Eklund, 1983).

Most investigations into the antimicrobial activity of lactate salts have been in meat and poultry products. When vacuum packaged pork loins were pumped with NaL, 1% and 2% NaL reduced aerobic plate counts (APC) by 1.46 and 1.49 $\log_{10}$ CFU/ml, respectively. When 2% NaL was combined with 0.2% sodium tripolyphosphate, APC's were reduced 2.5 $\log_{10}$ CFU/ml. This is attributed to the lowering of the pH in the product (Banks et al, 1998). It also indicated

that lactate salts can be used in combination with other antimicrobials to extend shelf life. When combined with 0.5% sodium chloride and 0.3% sodium tripolyphosphate in cooked beef top rounds, 4% NaL reduced APC's more than controls. NaL was also observed to be more effective at 4$^{o}$C than at 0$^{o}$C (Maca et al, 1999), possibly due to the higher kinetic activity at 4$^{o}$C. The application of NaL possibly increased the lag phase of bacteria. In another study, hamburger patties with 3 and 4% NaL alone reduced APC's significantly by day 3 and day 2 at 4$^{o}$C, respectively. When 3% NaL was used in combination with 0. 2 % sodium propionate, a 2.3 $\log_{10}$ CFU/ML reduction was achieved over controls, not significantly different from 4 % NaL (2.7 $\log_{10}$ reduction) acting alone (Eckert et al, 1997). The addition of 3 % NaL to reduced fat beef patties decreased APCs on aerobic and vacuum packaged patties (Kulshrestha and Rhee, 1996).

The effects of heating meat products in the presence of NaL have been investigated (McMahon et al, 1999). Minced beef was inoculated with *Yersinia enterocolitica* and *Listeria monocytogenes*, and 0, 2.4% and 4.8% NaL were also added. After heating at 55$^{o}$C, D-values for each bacteria were determined on *Yersinia* selective agar and Palcam agar (selective for *Listeria monocytogenes*) supplemented with the same percentage NaL at two different pH's – 5.7 and 7.4. No recovery of either bacteria was observed on media supplemented with 4.8% NaL at pH 5.7. There was a significant decrease in D-values between 0% and 2.4% NaL supplemented minced beef. Lower D- values for all treatments were also observed for both bacteria when plated on 2.4 % NaL supplemented agar (over unsupplemented agar). These results show that there is a synergistic inhibitory effect from the NaL/heat combination. The presence of the NaL lowered D-values for both organisms, and its continued presence inhibited recovery of the organisms (McMahon et al, 1999). The use of selective agars in this study may have influenced the recovery. These results contradict those found by Kotorola and Conner (1997) in a heating study with *E. coli* O157:H7. In ground turkey meat containing a ) 8 % NaCl, b) 4 % NaL, c) 0.4 % sodium polyphopshate, and d) a combination of these additives, D-values actually increased over the control (no additives) when heated to 55$^{o}$C (plate counts were performed on Phenol Red Agar with 1% sorbitol). It was postulated that the additives lowered the water activity of the meat, which simultaneously decreased the effectiveness of the additives. The different organisms and media substrates could have contributed to the discrepancy in the findings of these two studies.

Calcium lactate (CaL) has been used on various produce, but not solely as an antimicrobial. It was used as a firming agent on fresh cut cantaloupe cylinders, and provided a higher degree of firmness while imparting less bitterness than calcium chloride. No conclusive antimicrobial effect was shown in this experiment (Luna-Guzman and Barrett, 2000). Gorny et al. (1999) found no significant extension of shelf life when applying a 1 % CaL solution (with 2 % ascorbic acid) to fresh cut peaches and nectarines. The possible effects of the calcium ion in fruit shelf life extension cannot be overlooked. Apples that were treated with calcium chloride and stored for 6 months were less susceptible to *Penicillium expansum* invasion than those that were not (Conway and Sams, 1984). As opposed to a direct antimicrobial affect, this may be due to the increase in tissue firmness that the calcium provides, strengthening the pectin structure and making it more resistant to microbial invasion. Other additives have also been used as antimicrobials on produce – whole or fresh cut. Trisodium phosphate reduced *S.* Montevideo counts on the surface and in the core of tomatoes at concentrations of 10 and 15 % (Zhuang and Beuchat, 1996). If trisodium phosphate has this effect, then tricalcium phosphate, a common calcium supplement, may also have potential antimicrobial activity. Tricalcium phosphate's antimicrobial properties have been studied less extensively. Similarly, the shelf life of peeled oranges (whole and chunked) was extended when fruit was dipped in 0.5% or 1.0% citric acid, or when the peel was infused with a range of 0.1 to 1.0% citric acid. Regardless of treatment method, maximum shelf life was attained with 0.5% citric acid at 4$^{o}$C and 1.0% at 8$^{o}$C and 21$^{o}$C. This inhibited spoilage microorganisms on the surface of the fruit by lowering the pH (Pao and Petracek, 1997).

### *Calcium in the Diet*

Calcium is an important mineral in sustaining human health. It contributes to a variety of functions of the body, including maintaining skeletal integrity, regulating nerve excitability, muscle contraction and blood coagulation (Weaver, 1998). Calcium must be ingested in foods and supplements. Nearly all of the calcium (99%) in the body is contained in the skeleton (Weaver, 1998). In childhood and adolescence, calcium is instrumental in building bone mass and preventing skeletal disorders. Osteopenia, osteoporosis and increased risk of fractures are all consequences of inadequate calcium intake. Some studies also cite the as yet undefined role of calcium in reducing hypertension, gestational hypertension, hypercholesteremia and carcinogenesis (Levenson, 1994). The Dietary Reference Intake (DRI) for calcium,

recommended by the Food and Nutrition Board of the National Academy of Sciences, range from 210 mg/ day for infants to 1300mg/ day for young males and females. These numbers vary based on the sex and age of the population. Calcium intake between 1-2g / day is well tolerated among most individuals. Excessive calcium supplementation may lead to kidney stone formation, constipation, intestinal bloating and excess gas. Calcium can also obstruct the absorption of drugs and minerals such as salicylates, bisphosphonates, fluoride and iron (Levenson, 1994).

Dairy products provide the highest level of calcium. Other foods with high levels of calcium are bony fish, spinach, collards, tofu and grains. Foods that contain significantly lower levels of calcium include fruits, vegetables, pasta and breads. High amounts of oxalic acid and phytates can form insoluble complexes with calcium and not allow absorption in the intestines (Levenson, 1994). The presence of excessive phosphates (more than 2:1 phosphate: calcium) can also hinder absorption. Calcium dosages may also play a role in absorption. The same amount given in six separate doses was better absorbed than the total amount in a single dose. It is disputed if meal conditions (full meal vs. a fasting state) have an effect on absorption (Levenson, 1994). Most calcium preparations must dissociate into elemental calcium before being absorbed. Acidic pH's, like those in gastrointestinal system, promote the dissolution of the calcium salt. Calcium bioavailability is the amount of the element that can be assimilated by the body, relating to the absorption, transport and utilization of the nutrient (Weaver, 1998).

As food consumption patterns change in the U.S. and the population continues to consume less calcium in their diet, more foods are being fortified with the mineral. Rice, bread and soy milk have all been fortified with calcium lactate (Hettiarachchy et al, 1996, Ranhotra et al, 1997, Lihono et al, 1997). Fruit juices provide a good vehicle for calcium fortification because of their high rate of consumption by all demographic groups (Siega-Riz et al, 2000). They also provide a calcium source for that portion of the population that is lactose intolerant or who have allergies that prevent them from consuming dairy products. Recent studies show that carbonated beverage and fruit juice consumption rose among male and female teenagers, while milk consumption decreased (Harnack et al, 1999). Calcium fortification of orange juice serves the changing food consumption patterns of the population. In general, calcium fortified orange juice provides 350 mg / serving, while milk provides 300 mg / day of the DRI.

The most prevalent calcium supplement is calcium carbonate ($CaCO_3$). However, it is fairly insoluble, especially at a neutral pH (less than 0.1 % in water). This makes it a poor

choice to supplement orange juice, and it is not used in that capacity. Calcium citrate (sometimes referred to as tricalcium citrate) is more soluble than calcium carbonate but still rather essentially insoluble (approximately 0.1 % in water). Tricalcium phosphate has a rather high calcium content (Table 2) but is still rather insoluble (less than 0.1% in water). Tricalcium phosphate is used commercially to fortify orange juice, sometimes in conjunction with calcium lactate. Calcium lactate is more soluble, about 9 g/ 100 ml $H_20$, depending on the isomer. Calcium citrate malate (CCM) has an even higher degree of solubility than calcium lactate and is considered to be 10 times more soluble than calcium citrate, making it better suited for the fortification of orange juice. Solubilities have no relation to the bioavailability of calcium (Weaver, 1998), but they can affect the appearance of the product.

Positive calcium balance reflects that more calcium is ingested by the body than is excreted. Orange juice fortified with CCM was found to confer the highest positive calcium balance among participants taking calcium carbonate tablets, soda fortified with liquid calcium, milk, and cheese (Kohls, 1991). However, it was not determined whether this positive balance was a result of the delivery vehicle (orange juice) or the form (CCM).

TABLE 2**.** Commonly Used Calcium Supplements in Orange Juice

| Calcium supplement | Alternative names | Chemical formula | %Ca |
|---|---|---|---|
| Calcium citrate | Calcium citrate tetrahydrate<br>Tricalcium citrate tetrahydrate | $Ca_3(C_6H_5O_7)_2$-$4H_2O$ | 21 |
| Tricalcium phosphate | Calcium phosphate tribasic; hydroxyapatite; calcium hydroxide phosphate | $Ca_{10}(OH)_2(PO_4)_6$-$XH_2O$ | 34-40 |
| Calcium lactate | Calcium lactate pentahydrate;<br>Calcium lactate trihydrate; | $C_6H_{10}O_6Ca$-$5H_2O$ | 14 |
| Calcium citrate malate | Calcium citrate malate | 6 Ca: 2 Citrate: 3 Malate | 21 |

In rat and human studies, apple juice fortified with CCM was shown to have higher calcium absorption than CCM fortified orange juice (Andon et al, 1996).However, it was not determined whether this positive balance was a result of the delivery vehicle (orange juice) or the calcium form (CCM). In rat and human studies, apple juice fortified with CCM was shown to have higher calcium absoprtion than CCM-fortified orange juice. This could be due to the higher fructose content of apple juice than orange juice, indicating the carbohydrate composition of the juice plays a role in calcium absorption. It should also be noted that calcium absorption was relatively high from both juices, with over one-third of the ingested calcium being absorbed in both (Andon et al, 1996).

Calcium fortified orange juice can enhance the activity of the pectin methyl esterase (PME) enzyme. Calcium supplemented juices that were held at low temperature before heat treatment had PME activity, decreasing flavor characteristics, juice cloud density, and causing increased viscosity and settling pulp (Baker et al, 1991).

Although calcium supplements may be used for nutritional purposes, they might also affect the microbial ecology of orange juice. Pathogens like *Salmonella* that traditionally would not be considered a risk in a low pH product like orange juice might be sustained due to addition of a calcium supplement, or conversely, might die off more quickly. Potentially, these might prove to be effective interventions in the processing of unpasteurized juice. These investigations could also change the processing parameters of producing pasteurized orange juice, by increasing or decreasing thermal treatment times. It could also lead to a multifunctional calcium supplement – one that has both nutritional and antimicrobial benefits. As an increasing number of fruit juices are being fortified with calcium, it may help determine which supplement might have the greatest microbial impact. The studies presented here describe *Salmonella* in calcium fortified orange juice to determine a) which supplements enhance or diminish survival of the organism, and b) the variation in survivability among *Salmonella* serotypes.

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# CHAPTER 2

# SURVIVAL OF SALMONELLAE IN PASTEURIZED, REFRIGERATED CALCIUM-FORTIFIED ORANGE JUICE[1]

[1]Sharma, Manan, Larry R. Beuchat, Michael P. Doyle and Jinru Chen. To be submitted to Journal of Food Protection.

A study was undertaken to determine the survival of *Salmonella* in orange juice fortified with calcium. Strains of *S.* Muenchen (Inoculum 1) and serotypes of *Salmonella* from human and animal origin (Inoculum 2) and from raw produce and juice-associated outbreaks (Inoculum 3) were inoculated into commercially pasteurized orange juice, with or without added calcium. Inoculated juice initially contained populations of 6.61 to 6.98 $\log_{10}$ CFU/ml and was stored at 4$^{o}$C for up to 32 days. Juice was fortified with calcium at a concentration of 350-mg calcium/240 ml (1.46-mg calcium/ml), and non-fortified juices served as controls. The population of Inoculum 1 that survived in juice fortified with calcium lactate/tricalcium phosphate (CaL/TCP) was significantly lower ($p \leq 0.05$) (2.80 $\log_{10}$ CFU/ml) than in non-fortified juice (3.50 $\log_{10}$ CFU/ml) over 32 days. *Salmonella* counts in Inocula 1 and 2 declined less rapidly in juice fortified with TCP (3.21 and 3.33 $\log_{10}$ CFU/ml), respectively than in the non-fortified juice (3.75 $\log_{10}$ CFU/ml and 4.15 $\log_{10}$ CFU/ml), respectively. Over the storage period, populations in Inocula 1and 3 showed significantly slower rates of inactivation (2.62 $\log_{10}$ CFU/ml and 3.12 $\log_{10}$ CFU/ml) in juice fortified with calcium citrate (CC) than in non-fortified juice (3.14 $\log_{10}$ CFU/ml and 3.60 $\pm$ 0.20 $\log_{10}$ CFU/ml, respectively). There were no significant differences in the survival of *Salmonella* in juice fortified with calcium citrate malate (CCM) and non-fortified juice. PCR typing of randomly selected *Salmonella* colonies revealed that *S.* Heidelberg in Inoculum 2 and *S.* Baildon and *S.* Poona in Inoculum 3 were the most prevalent at the end of the 32-day storage period at 4$^{o}$C. This study reveals that form of calcium used to fortify orange juice can impact the survival of *Salmonella.*

Unpasteurized orange juice has been the vehicle for several outbreaks of salmonellosis. *Salmonella* Typhi was identified as the causative agent in an outbreak of infections in Ohio in 1944, which resulted in 18 cases of illness and 1 death (*6*, *12*). Consumption of unpasteurized orange juice contaminated with *S.* Typhi at a New York hotel in 1989 led to 67 cases of salmonellosis (*2*). The source of *Salmonella* in both of these incidents was most likely infected food handlers.

In 1999, 298 cases of salmonellosis in the United States and Canada were linked to unpasteurized orange juice containing *Salmonella* Muenchen (*3*). Oranges, the processing environment, storage and transport facilities were potential sources of the pathogen. In 1995, 63 confirmed cases of salmonellosis were associated with the consumption of unpasteurized orange juice containing *Salmonella* Hartford at a Florida theme park, although estimates of up to 6,300 persons were infected (*5*). The source of *S.* Hartford was amphibians that introduced the pathogen into the orange juice processing facility (*11*).

These outbreaks indicate that *Salmonella* can survive refrigerated, acidic conditions for a sufficient time and at a population high enough to cause illness. Unpasteurized orange juice presents a greater public health risk than pasteurized juice because of the lack of a physical or chemical intervention to destroy pathogenic microorganisms. *Salmonella* has been shown to grow on the surface of freshly peeled oranges at 24$^{o}$C (*10*). Oranges, when placed in a cold inoculum of *Escherichia coli* O157: H7, internalized the pathogen (*16*). Vigilant plant sanitation and close monitoring of transport and storage temperatures are currently utilized to minimize contamination and potential growth of pathogens in unpasteurized juice.

Fruit juices fortified with calcium have become increasingly popular in recent years. Orange juice is fortified to provide 35% (350 mg) of the Dietary Reference Intake (DRI) value of calcium per 240-ml (8-oz.) serving. Various calcium salts are used to fortify orange juice, including calcium lactate, a calcium lactate/tricalcium phosphate combination, tricalcium phosphate, calcium citrate malate, and calcium citrate. However, calcium fortification of orange juice has not been assessed to determine its effect on survival or growth of *Salmonella* in orange juice.

The objectives of this study were to evaluate the effect of calcium supplements on survival of salmonellae in orange juice at 4$^{o}$C over an extended period of time and to determine if *S.* Muenchen displayed unique survival characteristics in orange juice when compared with *Salmonella* isolates from patients with salmonellosis associated with other sources.

# MATERIALS AND METHODS

**Orange juice**. Four brands of pasteurized orange juice were purchased from a local supermarket. Calcium-fortified and non-fortified juice of each brand were evaluated. Brand A was fortified with a combination of calcium lactate (CaL) and tricalcium phosphate (TCP) salts and Brand B contained orange juice fortified with calcium citrate malate (CCM). Tricalcium phosphate (TCP) was used as the fortificant in Brand C, while Brand D was fortified with calcium citrate (CC). All calcium-fortified juices provided 350 mg/calcium per 240 ml (8 oz.). Juices were selected so that the only difference between calcium-fortified and non-fortified juices in the same pair was the presence of the calcium supplement listed on the ingredient label. Brand A and Brand C contained orange juice made from concentrate. Juices were stored at 4$^{o}$C for 2 days until used.

**Strains used**. Three five strain *Salmonella* inocula were used. Inoculum 1 contained five *S.* Muenchen isolates, provided by Dr. Ramesh Gauton at the State of Washington Department of Health, Seattle, WA. Inoculum 2 contained, *S.* Typhimurium, *S.* Heidelberg, *S.* Thompson, *S.* Infantis, and *S.* Enteritidis from human or animal sources. Serotypes implicated in produce-associated outbreaks were used to prepare Inoculum 3: *S.* Gaminara (orange juice), *S.* Hartford (orange juice), *S.* Michigan (cantaloupe), *S.* Baildon (tomato), and *S.* Poona (cantaloupe).

Stock cultures of *Salmonella* were inoculated on BHIA (Brain Heart Infusion Agar, Difco Laboratories, Detroit, Mich.) plates and incubated at 37$^{o}$C for 24 h. Cultures were then transferred on to fresh BHIA plates under the same conditions. A single colony of each strain was then inoculated into 10 ml of BHI broth (Difco) and incubated at 37$^{o}$C with agitation (165 rpm) until cell populations reached an optical density (O.D.) at 600 nm of 0.90-95. After reaching this O.D., strains were placed at 4$^{o}$C until used. Inocula were prepared by combining five *S.* Muenchen strains or five serotypes. Each five-strain or five-serotype mixture (5 ml) was sedimented by centrifugation (2,720 x *g* for 20 min at 20$^{o}$C). The supernatant fluid was decanted, and the cell pellet was suspended in 5 ml of the same type of orange juice into which it would be used as an inoculum. Each inoculum (1 ml) was added to 99 ml of orange juice. Initial populations of *Salmonella* in inoculated orange juice were ca. $10^{7}$ CFU/ml. Inoculated juice was mixed thoroughly and held at 4$^{o}$C for up to 32 days.

**Analysis of orange juice**. Juice was analyzed 15 times over the 32-day storage period. Immediately before juice was analyzed, it was mixed for 10 s. One milliliter of juice was withdrawn, serially diluted 1:10 in sterile 0.1% peptone water and plated (0.1 ml, in duplicate) on Bismuth Sulfite Agar (BSA, Difco). Plates were incubated at 37°C for 24 h before colonies were counted. Two replicate experiments were conducted.

**Statistical Analysis**. Data were analyzed using multiple regression analysis in SAS software (SAS Institute, Cary, NC). Plate counts ($\log_{10}$ CFU/ml) were plotted against time (days) of storage at 4°C within each brand. The slope of the survival curve for *Salmonella* in calcium-fortified orange juice was compared to the slope in non-fortified juice. Slopes of survival curves for all inocula in each orange juice were also compared for significant differences.

**PCR Analysis of *Salmonella* serotypes**. *Salmonella* colonies isolated on BSA from juice stored for 32 days at 4°C were subjected to PCR analysis to determine if one or more strains predominated. Five randomly selected colonies from each of the eight juices inoculated with Inoculum 2 or Inoculum 3 of the storage study were streaked on Tryptic Soy Agar (TSA, Difco Laboratories) plates and incubated at 37°C for 24 h. Cells from colonies were transferred to 200 µl of Tryptic Soy Broth (TSB, Difco) in a 1.5-ml micro-centrifuge and incubated at 37°C for 16-18 h. Cultures were centrifuged at16,000 x *g* for 3 min. Supernatant was decanted, and cell pellets were washed twice in 200 µl sterile deionized water. After the final wash, the pellet was resuspended in 200 µl of deionized water and heated in a boiling water bath for 10 min, followed by centrifugation at 16,000 x *g* for 10 min. DNA in the supernatant was used as template in PCR amplification. The reaction mixture consisted of 13 µl of sterile $H_20$, 5 µl of 10 X PCR buffer, 3 µl of $MgCl_2$ (25 mM), 6 µl of dNTP's (10mM), 20 µl of template DNA, 2 µl of ERIC2 primer (*8*), and 1 µl of *Taq* DNA polymerase (1 U/µl) were used. All molecular reagents were supplied by Roche Molecular Biochemicals (Indianapolis, Ind.). Reactions were carried out in a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, Conn.) under the following conditions: 94°C for 5 min (1 cycle), 92°C for 45 s, 25°C for 1 min, 68°C for 10 min (30 cycles), and 72°C for 20 min (1 cycle), then held at 4°C. PCR products were then separated on 1% agarose gel. Following electrophoresis, gels were stained in ethidium bromide solution (1 µg/ml) and viewed and photographed using the Gel Doc 2000 system (BioRad, Hercules, Calif.).

Cluster analysis of PCR fingerprints of *Salmonella* strains was performed using 1D Advantage software (Advanced American Biotechnology, Fullerton, Cali.).

## RESULTS

**Survival of salmonellae in orange juice.** Initial *Salmonella* populations (day 0) in orange juice inoculated with Inocula 1, 2 and 3 in orange juice samples ranged from 6.69 to 6.93 $\log_{10}$ CFU/ml, 6.61 to 6.77 $\log_{10}$ CFU/ml, and 6.83 to 6.98 $\log_{10}$ CFU/ml, respectively. Figure 1 shows populations of *S.* Muenchen in orange juice containing Inoculum 1. Counts in Brand A juice fortified with CaL/TCP declined at a significantly faster rate ($p \leq 0.05$) compared to the non-fortified control. The presence of CCM in Brand B juice did not influence the rate of death of *S.* Muenchen. However, populations in Brand C juice containing TCP and Brand D juice containing CC declined at significantly slower rates than those in non-fortified controls.

Shown in Figure 2 are survival curves for salmonellae in Inoculum 2. Brand A fortified with CaL/TCP and Brand B fortified with CCM did not influence the rate of inactivation. Juice fortified with TCP (Brand C) enhanced the survival of serotypes in Inocula 2, but juice fortified with CC (Brand D) did not have a significant effect on the survival when compared with non-fortified controls.

Calcium-fortified juices in Brand A (CaL/TCP), Brand B (CCM), and Brand C (TCP) exerted no more of an effect on the survival of serotypes of in Inoculum 3 than non-fortified controls (Figure 3). Similar to Inoculum 1, juice fortified with CC in Brand D enhanced the survival of Inocula 3 over the non-fortified control.

**Comparison of different *Salmonella* inocula.** With the exception of Brand A orange juice, the three test inocula behaved similarly in calcium-fortified and non-fortified juices. Compared to salmonellae in Inocula 2 and 3, *S.* Muenchen declined significantly more slowly in the non-fortified control of Brand A.

**PCR analysis results**. Thirty-eight isolates from orange juices inoculated with Inoculum 2 and 40 isolates from juice inoculated with Inoculum 3 were analyzed using PCR to determine if one or more serotypes predominated at the end of the 32-day storage period. PCR fingerprints indicate that *S.* Heidelberg was prevalent in 24 of 38 (63%) isolates from juice inoculated with Inoculum 2 (Table 1). Nine of the 38 colonies (24%) corresponded to the banding pattern of *S.* Enteritidis. *S.* Thompson and *S.* Infantis matched the PCR fingerprint for

8% (3/38) and 5% (2/38) of the colonies tested, respectively. None (0/38) of the colonies tested matched the banding pattern of *S.* Typhimurium. For Inoculum 3 isolates, 43% (17/40) of test colonies had the same banding pattern as *S.* Baildon, while 35% (14/38) matched the same fingerprint as *S.* Poona. The banding pattern of 23% (9/40) of the colonies matched that of *S.* Hartford. None (0/38) of the tested colonies were consistent with the fingerprint of *S.* Gaminara or *S.* Michigan.

## DISCUSSION

Calcium lactate may act as an antimicrobial in orange juice to lower *Salmonella* counts. No antimicrobial activity was observed in juice fortified with TCP, indicating that the principal antimicrobial activity can be attributed to CaL in juice fortified with CaL/TCP. The low pH of orange juice enhances the effect of CaL as an antimicrobial in orange juice by allowing the undissociated, non-polar form of the lactate ion ($CH_3CH_2OHCOOH$) to cross the bacterial cell membrane and acidify the interior of the cell. The presence of the lactate ion disrupts the hydrogen ion efflux from the bacterial cell and leads to cell death (*14*). Sodium lactate salts have been shown to reduce aerobic plate counts on a variety of meat products (*1*, *7*, *9*).

The addition of lactic acid (0.1%) to unpasteurized apple cider inoculated with *Escherichia coli* O157: H7 has been reported to result in a 5-$\log_{10}$ CFU/ml reduction after subjecting the cider to freezing (48 h at –20$^{o}$C), thawing (4 h at 4$^{o}$C), and holding for 6 h at 35$^{o}$C (*15*). Calcium lactate also has the potential for use in combination with physical or other chemical treatments to reduce pathogen populations in unpasteurized fruit juices.

Orange juice fortified with TCP enhanced survival of *S.* Muenchen in Inocula 1 and serotypes from humans and animals in Inocula 2. The pH of the non-fortified juice in Brand B decreased more than the pH of juice fortified with TCP during the 32-day storage period (Table 2). The chemical structure of TCP is $Ca_{10}(OH)_2(PO_4)_6$-$XH_2O$ (X is undetermined). This structure contributes hydroxide ions that raise the pH of the orange juice. Salmonellae encountered less acid stress in juice fortified with TCP compared to non-fortified juice and retained higher viability. These results are in agreement with a previous study (*13*), which found that acid adapted salmonellae survived for 27 days in orange juice at pH 3.5, while salmonellae in orange juice at pH 4.4 survived for 73 days. TCP also raised the pH of orange juice fortified with CaL/TCP, but CaL still displayed antimicrobial properties.

Similar to juice fortified with TCP, the decreased acid stress encountered by salmonellae in juice fortified with CC may have enhanced survival over those cells in non-fortified juice. The addition of CC, when combined with citric acid, creates a buffering effect in the orange juice. This decreases the free hydrogen ion concentration in the juice and increases the pH.

No single commercial juice manufacturer uses all four types of calcium supplements evaluated in this experiment. This makes it difficult to compare the survival of *Salmonella* as influenced by calcium salts used for fortification. Differences in the composition of juices, packaging, and storage and transport conditions may also affect the survival of *Salmonella* in commercially fortified orange juices.

No consistent relationship was established in patterns of survival of cells of mixed serotypes of *Salmonella* in inocula. This suggests that the environmental source of *Salmonella* isolates plays a minimal role in determining survival characteristics in orange juice.

*S.* Heidelberg was the most prominent serotype in juices containing Inoculum 2 after storage for 32 days. *S.* Baildon and *S.* Poona were the predominant serotypes in juices containing Inoculum 3. *S.* Baildon was implicated in an outbreak of salmonellosis associated with diced tomatoes (personal communication). It has been suggested that the *S.* Baildon isolate from this outbreak may be intrinsically more resistant to acid stress than other serotypes of *Salmonella* (*17*). There were 400 culture-confirmed infections caused by *S.* Poona in an outbreak involving the consumption of cantaloupe (*4*). Serotypes in Inoculum 3 that were previously associated with unpasteurized orange juice (*S.* Hartford and *S.* Gaminara) were not as prevalent as *S.* Baildon and *S.* Poona at the end of 32 days of storage. Further investigation into acid tolerance of *S.* Heidelberg, *S.* Baildon, and *S.* Poona is warranted.

In summary, the fortification of orange juice with calcium affected the survival of *Salmonella.* Calcium lactate/tricalcium phosphate supplements increased the rate of inactivation of *S.* Muenchen, while calcium citrate and tricalcium phosphate slowed the rate of inactivation of *Salmonella* in some cases. Using CaL in unrefrigerated fruit juices could affect a more rapid reduction in spoilage microorganisms because the efficacy of CaL at temperatures higher than $4^{o}$C would be enhanced. Concentrations of CaL in orange juice may also be modified to enhance antimicrobial activity, but may alter the nutritional or sensory characteristics of fortified orange juice. Survival of *Salmonella* as affected by calcium fortification should be investigated to compare the effect of various calcium salts in a single brand of orange juice.

Further investigation into the effect of calcium lactate on pathogenic and spoilage microorganisms in orange juice would benefit both consumer and commercial interests.

## ACKNOWLEDGMENTS

We would like to thank Mr. Jerry Davis for providing statistical analysis and support, and Dr. Ying Mao, Dr. Mahbub Islam, and Ms. Joy Adams for technical support.

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TABLE 1. *Serotype distribution of* Salmonella *in orange juices stored at 4°C for 32 days.*

| Serotype | Brand A[a] | | Brand B | | Brand C | | Brand D | | Total[b] |
|---|---|---|---|---|---|---|---|---|---|
| | NF | CaL/TCP | NF | CCM | NF | TCP | NF | CC | |
| Inoculum 2 | | | | | | | | | |
| *S.* Typhimurium | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S.* Heidelberg | 2 | 4 | 2 | 3 | 2 | 3 | 3 | 5 | 24 |
| *S.* Thompson | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 3 |
| *S.* Infantis | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 2 |
| *S.* Enteritidis | 3 | 0 | 2 | 1 | 2 | 0 | 1 | 0 | 9 |
| Inoculum 3 | | | | | | | | | |
| *S.* Gaminara | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S.* Michigan | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S.* Baildon | 0 | 4 | 4 | 2 | 3 | 1 | 0 | 3 | 17 |
| *S.* Hartford | 1 | 0 | 0 | 1 | 1 | 4 | 1 | 2 | 9 |
| *S.* Poona | 4 | 1 | 1 | 2 | 2 | 0 | 4 | 0 | 14 |

[a] NF, non-fortified; CaL/TCP, calcium lactate/tricalcium phosphate; CCM, calcium citrate malate; TCP, tricalcium phosphate; CC, calcium citrate

[b]Number out of 38 colonies from juices containing Inoculum 2 and 40 colonies from juices containing Inoculum 3

TABLE 2. *The initial and final pH of orange juices stored at 4°C for 32 days*

| Orange juice | | pH | |
|---|---|---|---|
| Brand | Treatment[a] | Initial | Final |
| A | Non-fortified | 3.98 | 3.93 |
| | CaL/TCP | 4.28 | 4.20 |
| B | Non-fortified | 4.07 | 3.94 |
| | CCM | 4.24 | 4.15 |
| C | Non-fortified | 4.02 | 3.79 |
| | TCP | 4.07 | 4.00 |
| D | Non-fortified | 3.91 | 3.85 |
| | CC | 4.13 | 4.05 |

[a] Juice was not fortified with calcium or fortified with calcium lactate and tricalcium phosphate (CaL/TCP), calcium citrate malate (CCM), tricalcium phosphate (TCP), calcium citrate (CC).

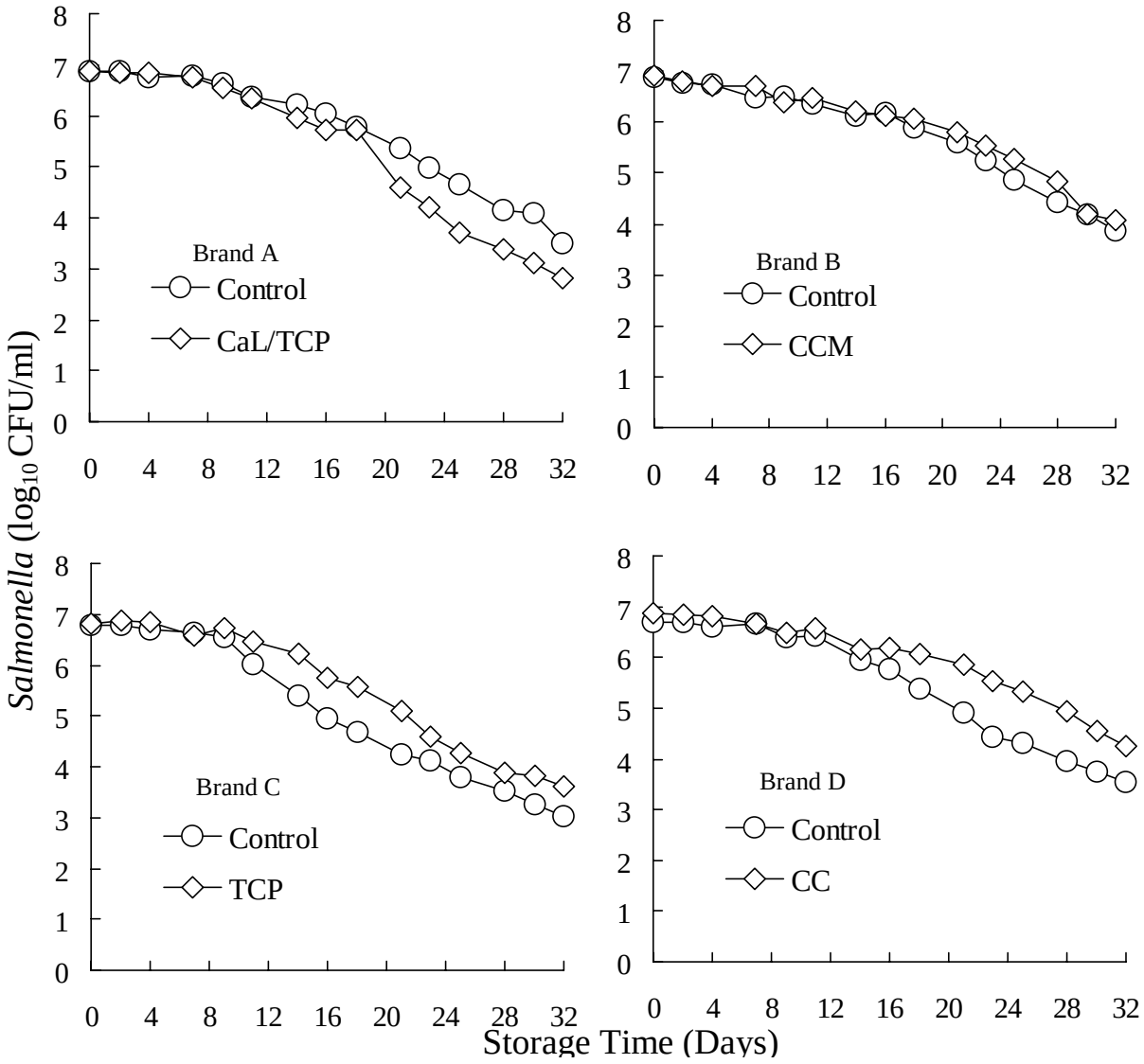


FIGURE 1. *Survival of* Salmonella *from human and animal origin (Inoculum 1) in orange juice stored at 4°C for 32 days.*

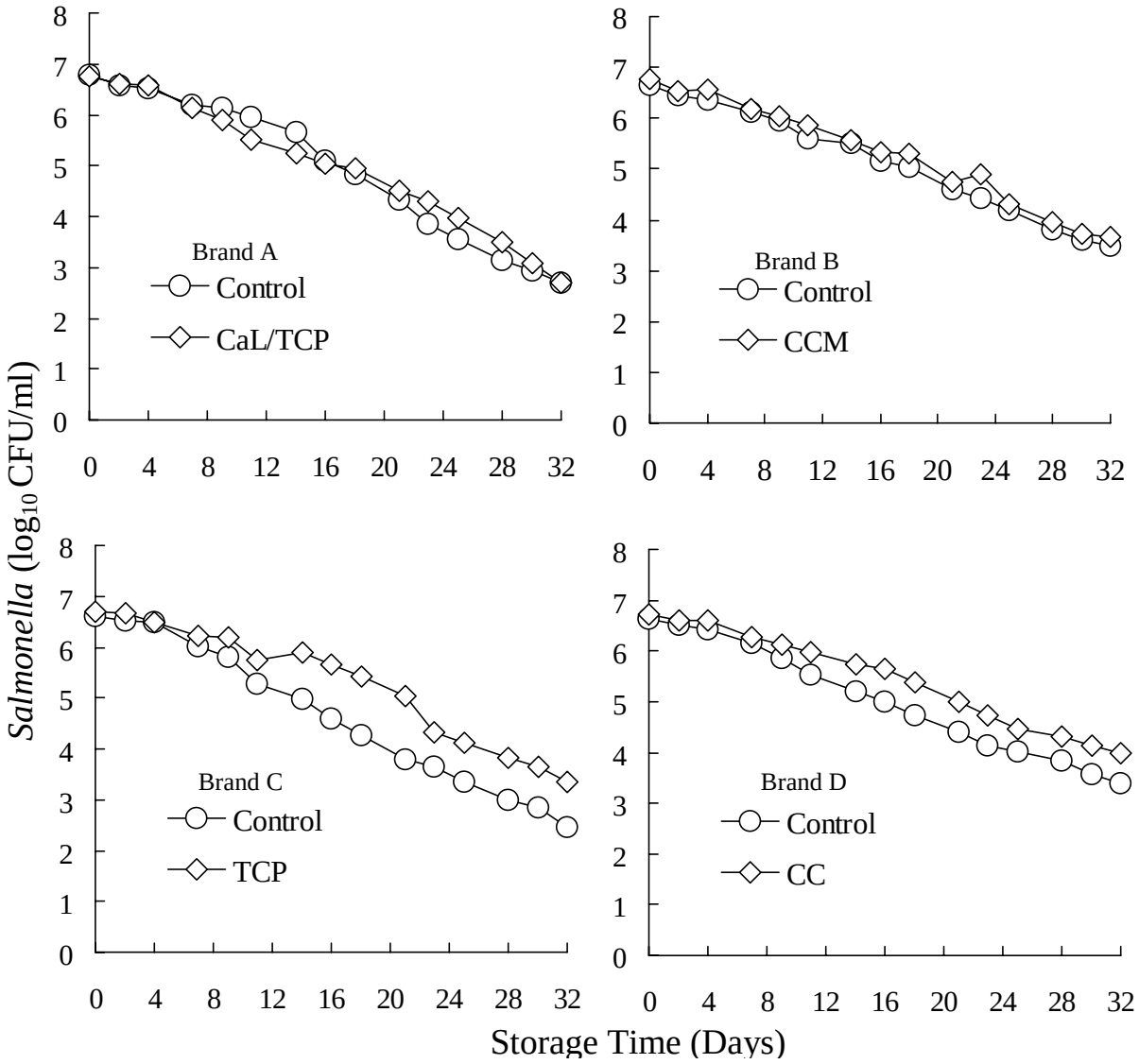


FIGURE 2. *Survival of* Salmonella *from human and animal origin (Inoculum 2) in orange juice stored at 4°C for 32 days.*

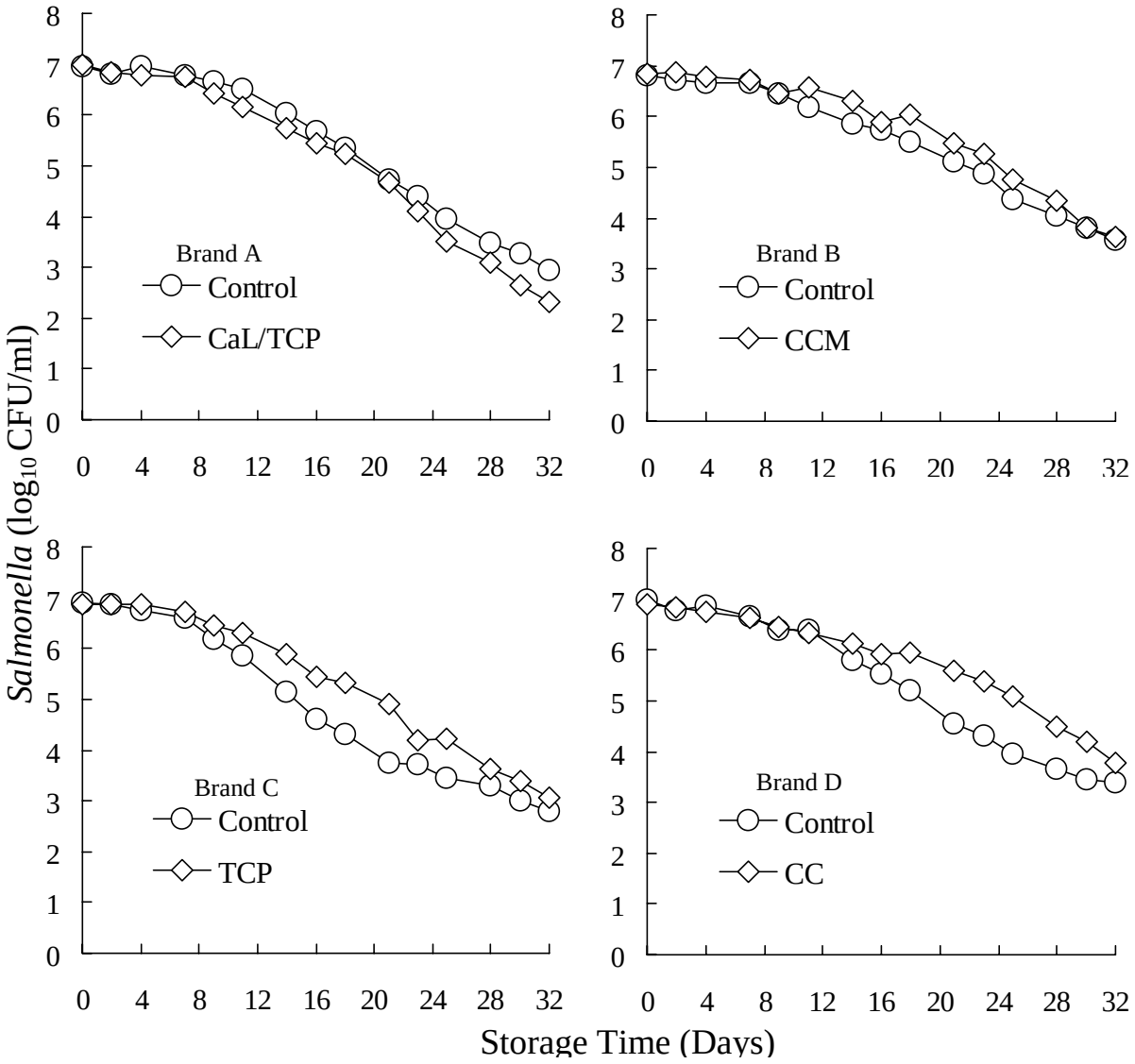


FIGURE 3. *Survival of* Salmonella *from produce-associated outbreaks (Inoculum 3) in orange juice stored at 4°C for 32 days.*

# CHAPTER 3

# FATE OF SALMONELLAE IN CALCIUM-SUPPLEMENTED ORANGE JUICE AT REFRIGERATION TEMPERATURE[1]

[1]Sharma, Manan, Larry R. Beuchat, Michael P. Doyle and Jinru Chen. To be submitted to Applied and Environmental Microbiology.

Recent outbreaks of salmonellosis associated with orange juice have raised interest regarding the survival and growth of *Salmonella* spp. in juice supplemented with calcium. A study was done to determine the influence of different calcium supplements on the survival of *Salmonella* spp. in orange juice held at 4$^{o}$C for up to 32 days. Isolates of *S.* Muenchen (Inoculum 1), of *Salmonella* spp. from humans and animals (Inoculum 2), and *Salmonella* spp. 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 calcium per 240-ml serving (1.46 mg calcium/ml). *Salmonella* populations 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 inoculum used as inoculum. Counts decreased from 4.89 $\log_{10}$ CFU/ml to < 1 $\log_{10}$ CFU/ml of orange juice supplemented with CaL and tricalcium phosphate (TCP) within 30 days. These reductions were significantly ($p \leq 0.05$) greater than that of the control (no calcium added), in which populations decreased 3.19 $\pm$ 0.20 $\log_{10}$ CFU/ml over 32 days. Populations in orange juice containing TCP and calcium citrate (CC) declined 1.34 $\pm$ 0.20 $\log_{10}$ CFU/ml and 1.96 $\pm$ 0.20 $\log_{10}$ CFU/ml, respectively, over 32 days. These counts were significantly higher than the control counts in juice stored for 32 days. Populations of *Salmonella* of Inoculum 3 inoculated into juice containing calcium citrate malate (CCM) were significantly greater than the control. Cells in Inoculum 3 also survived in higher populations than cells in Inoculums 1 or 2 in juice supplemented with CCM. Growth of *Salmonella* did not occur on tryptic soy agar supplemented with CaL or CaL/TCP and adjusted to pH 4. 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 non-typhoid *Salmonella* and is characterized by nausea, vomiting, abdominal pain, headaches, elevated body temperature, and non-bloody diarrhea. It can be fatal in severe cases. Most affected by the disease are those who are immunocompromised, children with underdeveloped immune systems, the elderly, who produce decreased amounts of stomach acid, and those who suffer from chronic illnesses (21).

In the summer of 1999, an outbreak of infections caused by *Salmonella* Muenchen occurred in western United States and Canada, in which unpasteurized orange juice was the vehicle of transmission (3). This outbreak resulted in 298 confirmed cases of salmonellosis and 1 death. Potential sources of contamination included oranges, the processing plant environment, and storage and transport facilities, although the source of contamination was not identified. This was the first reported outbreak of salmonellosis associated with unpasteurized orange juice implicating *S.* Muenchen, although other serotypes have been associated with previous outbreaks involving orange juice. *S.* Typhi was implicated in an outbreak in 1944 in Ohio, in which there were 18 cases with 1 death (6, 18). *S.* Typhi was again the causative agent in 1989 in an outbreak of 67 cases of salmonellosis linked to orange juice consumed at a New York hotel (1). Infected food handlers were sources of the pathogens in both of these outbreaks.

In 1995, *S.* Hartford contamination in unpasteurized orange juice consumed at a Florida theme park was epidemiologically linked to 62 confirmed infections, although estimates of up to 6300 persons were infected (4). A probable source of this contamination was amphibians that carried the pathogen into the juice processing facility (17).

Salmonellosis associated with consumption of unpasteurized orange juice suggest 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 (25). Growth of *E. coli* O157: H7 and *Salmonella* on surface-inoculated freshly peeled oranges has been observed (16). 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 upon to prevent the growth of pathogens in unpasteurized juice.

Calcium fortification of fruit juices has become increasingly popular in recent years. Commercially available orange, grapefruit, and grape juice, as well as lemonade and fruit

punch, are fortified with various forms of calcium to increase their nutritional value. As fruit juice and soft drink consumption increase and dairy product consumption decreases in the United States (10), juice can be an effective delivery vehicle for calcium. These products also provide an alternative calcium source for lactose intolerant individuals who have difficulty consuming dairy products. However, it is not known what effect calcium supplements have on the microbial ecology of orange juice. The calcium content in 240 ml (8 oz.) of calcium-supplemented orange juice provides 35% (350 mg) of the Dietary Reference Intake (DRI) value for calcium. Various forms of calcium, including a calcium lactate/tricalcium phosphate combination, tricalcium phosphate, calcium citrate, and calcium citrate malate are used to fortify juices. Sodium lactate salts have shown antimicrobial activity in various meat products (2, 12, 14) and trisodium phosphate is lethal to *Salmonella* on tomatoes (26). Based on these findings, calcium lactate and tricalcium phosphate could have potential antimicrobial activity in orange juice.

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 *S.* Muenchen, a serotype previously associated with illness from consumption of orange juice, has unusual survival characteristics in orange juice compared to other *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 calcium lactate (CaL), calcium citrate (CC), both supplied by Jungbunzlauer (Newton Centre, Mass.), tricalcium phosphate (TCP) (FMC, Lawrence, Kan), and calcium citrate malate (CCM), formulated in the laboratory according a procedure described by Fox et al (8). CCM was prepared by dissolving 192 g of citric acid (Fisher Scientific, Fair Lawn, N.J.) in 1000 ml of deionized water. Malic acid, DL form (201 g) (Sigma, St. Louis, Mo.), was added, and the solution was stirred until it appeared clear. Calcium carbonate (300 g) (J.T. Baker, Phillipsburg, N.J.) was slowly added to the solution and stirred for 3 h at 22$^{o}$C. Periodically, the solution was shaken to suspend the white precipitate on the bottom of the flask. The solution was poured into a sterile, rectangular pan (53 cm x 33 cm). The pan was placed in a convection oven preheated to 80$^{o}$C, and dried for 19 h. After cooling to 22$^{o}$C, the CCM was removed from the pan and ground in a Wiley mill

(Arthur H. Thomas Company, 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 for less than 48 h until used. This is referred to as the orange juice base.

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

A frozen stock culture of each strain was streaked on to tryptic soy agar (TSA; Becton Dickinson, Sparks, Md.) and incubated at 37°C for 24 h. Individual colonies of each strain were streaked on fresh TSA plates and incubated under the same conditions. Single colonies from each plate were then inoculated into 10 ml of tryptic soy broth (TSB; Becton Dickinson). Strains were incubated overnight at 37°C with agitation (170 rpm) until cell populations reached an optical density (O.D.) at 600 nm of 0.90 - 0.95. This O.D. corresponds to ~$10^9$ cfu of *Salmonella*/ml. Strains were stored at 4°C until used. To prepare inocula, 1 ml of culture of each of five strains 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 x *g* for 20 min at 20°C), 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 of 350 mg per 240-ml (8-oz.) serving, or 1.46 mg calcium/ml. A CaL/TCP treatment and a control with no calcium salt added were also tested. To 100 ml of orange juice, the following amounts of each salt were separately added: 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 bottles and 99 ml of orange juice base was added. The mixture was shaken to dissolve calcium salts. Most salts went into solution easily, but some of the TCP remained in suspension. Juice was stored at 4°C overnight until inoculation.

**Inoculation of orange juice**. Each of the three *Salmonella* inocula was serially diluted to $10^{-2}$ in orange juice base before inoculating into calcium-supplemented juice. Diluted suspension (1 ml) was added to 99 ml of each calcium-supplemented orange juice or the control orange juice (no calcium added). The initial population of *Salmonella* in inoculated orange juice was ca.$10^5$ CFU/ml.

**Analysis of orange juice**. Inoculated juice was mixed and stored at 4$^o$C for 4 h. The inoculated juice in each bottle was shaken by hand for approximately 10 s before samples were withdrawn to determine the population of *Salmonella.* Juice (1 ml) was diluted in 9 ml of sterile 0.1 % peptone water and surface plated (0.1 ml, in duplicate) on bismuth sulfite agar (BSA; Becton Dickinson). Plates were incubated at 37$^o$C for 24 h, and presumptive *Salmonella* colonies were enumerated. Uninoculated orange juice was also surface plated on BSA. Samples (0.25 ml) were plated on four BSA plates at each sampling time. Orange juice was analyzed for populations of *Salmonella* 15 times during 32 days of storage at 4$^o$C. Two replicate experiments were performed.

**Effect of calcium supplements on survival and growth on TSA**. TSA (500 ml) was brought to a boil and placed in a water bath at 70$^o$C. Each of the five calcium supplements was added in appropriate amounts for a concentration of 1.46 mg of calcium/ml. The pH of TSA containing each supplement was adjusted to 4.0 with 10 N hydrochloric acid or pH 7.0 with 5 N sodium hydroxide. TSA at pH 4.0 and 7.0 with no added calcium served as controls. All *Salmonella* inocula was serially diluted 1:10 in 0.1 % peptone water and was surface plated (0.1 ml, in duplicate) on each type of calcium supplemented TSA at each pH. Plates were incubated at 37$^o$C for 24 h before colonies were counted. Three replicate experiments were performed

**Analysis of data.** Data were analyzed using multiple regression analysis with SAS software (SAS Institute, Cary, N.C.). *Salmonella* counts ($\log_{10}$ CFU/ml) were plotted against days of storage at 4$^o$C. The slope of the survival curve for each inoculum in each orange juice sample was compared to that of the same inoculum inoculated into 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.

Populations of *Salmonella* in each inoculum plated on TSA with each calcium supplement were compared against the TSA control at each pH for statistically significant differences ($p \leq 0.05$).

# RESULTS

**Storage study.** *Salmonella* counts on BSA revealed that initial (day 0) populations 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 of the different inocula of *Salmonella* in orange juice with different calcium supplements stored at 4$^{o}$C for 32 days are shown in Figure 1. Salmonellae were inactivated most rapidly in juice containing CaL, with counts decreasing from 4.83 to < 1 $\log_{10}$ CFU/ml within 16 days. *Salmonella* were inactivated less rapidly in juice containing CaL/TCP, decreasing from 4.75 $\log_{10}$ to < 1 $\log_{10}$ CFU/ml within 28-30 days. Rates of decrease in *Salmonella* populations in juices supplemented with CaL and the CaL/TCP were significantly greater ($p \leq 0.05$) than in the control juice for all inocula. *Salmonella* counts decreased by 3.19 $\pm$ 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 1.34 $\pm$ 0.20 $\log_{10}$ CFU/ml during 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 $\log_{10}$ 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 $\log_{10}$ CFU/ml during 32 days. The rate of *Salmonella* inactivation in juice inoculated with Inoculum 3 was significantly slower than that of the control; however, death of *Salmonella* in orange juice inoculated with Inoculum 1 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.

**Growth on calcium-supplemented TSA.** TSA supplemented with CaL or CaL/TCP at pH 4 did not support *Salmonella* growth in any of the test inocula (Table 1). These counts (< 2 log CFU/ml) were significantly less than that in the control at pH 4 for all inocula (Table 1). TSA containing CC supported populations that were significantly higher than the control inoculated with Inoculum 1. At pH 7, no significant differences in the number of *Salmonella* recovered from Inoculum 1 on TSA with any calcium supplement and the control were observed. There were no significant differences in populations recovered on TSA (pH 4.0) containing CC, CCM, or TCP and TSA containing no added calcium (pH 4.0) for Inoculum 2.

Populations in Inoculum 2 plated on TSA with CC or CCM at pH 7 were significantly higher than populations recovered on control TSA. Populations of *Salmonella* in Inoculum 3 recovered on TSA supplemented with CC or CCM at pH 4 were significantly higher than those recovered on the control TSA, but populations on TSA containing TCP (pH 4.0) were significantly lower than those on the control. The presence of calcium salts in TSA at pH 7.0 did not have a significant influence on colony formation by *Salmonella* in Inoculum 3.

## DISCUSSION

Calcium lactate was most inhibitory to *Salmonella* of the calcium salts evaluated. 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 due prinicipally to the lactate ion. The efficacy of calcium lactate was somewhat unexpected considering that juice was stored at $4^{o}$C, which limits the overall kinetics of lactate penetration into bacterial cells.

Calcium lactate has been applied to stored, refrigerated fruit and fresh-cut fruit as a firming agent and to retain color (9, 13). 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 $pK_a$ of lactic acid, the conjugate acid of CaL, is 3.86 (22), which is the pH at which the undissociated form of the salt ($CH_3CHOHCOOH$) is in equilibrium with that of its dissociated form ($CH_3CHOHCOO^-$). Although the pH of the orange juice, 3.96, was greater than 3.86, it was low enough to allow a sufficiently high concentration of the non-polar, 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 (22). Many studies have examined the effect of sodium lactate salts on meat products. Beef top rounds treated with 4 % sodium lactate (NaL) and stored for 21 days had consistently lower aerobic plate counts (APCs) compared to the control (14). The addition of NaL to reduced-fat beef patties reduced APCs from 4.85 $\log_{10}$ CFU/ml to 2.70 $\log_{10}$ CFU/ml (12). Pork loins pumped with 1% and 2 % NaL reduced APCs by about 33% compared to the untreated control (2).

Calcium lactate is used commercially as a sole source of calcium to supplement orange juice at a concentration of 300 mg of calcium/240-ml serving (1.25 mg calcium/ml), less than the concentration used in this study (1.46 mg calcium/ml). A combination of CaL/TCP is also

used commercially to supplement orange juice but not in the same ratio 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. Reduction in the population of *Salmonella* observed in a preliminary study using a commercial orange juice labeled as containing CaL/TCP at a concentration sufficient to supply 35% of the DRI for calcium (350 mg/240 ml) was less than the reduction reported here, suggesting that the concentration of CaL in the commercially-supplemented juice was lower than that in juice supplemented with CaL/TCP used in this experiment.

Lower concentrations of CaL might be used without substantially compromising antimicrobial effectiveness. Calcium lactate could also be added to unpasteurized juices as part of a combination of physical and chemical interventions to provide a hurdle effect to kill or control the growth of pathogens in fruit juices. Lactic acid (0.1%) has been shown to decrease *E. coli* O157:H7 counts by $\geq$ 5 $\log_{10}$ CFU/ml of unpasteurized apple cider after freezing ( 48 h at –20$^{o}$C), thawing (4 h at 4$^{o}$C) and holding for 6 h at 35$^{o}$C (24).

Although pathogen reduction as a result of supplementation with CaL may not be a priority in unpasteurized or pasteurized juices, CaL treatments may nevertheless have the potential to reduce heat tolerance of bacterial and mold spores. The effectiveness of CaL in sensitizing spores to heat treatments used to pasteurize of fruit juices needs to be investigated. The use of CaL in unrefrigerated or heated fruit juices could potentially result in a more rapid reduction of vegetative cells and spores because of the increased ability of the lactate molecule to penetrate the cell at elevated temperatures. Several studies have shown accelerated death *of E. coli* O157: H7 by temporary storage of low pH food products at room temperature (15, 20).

Additional work is also 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 different fruit juices with different flavor characteristics, these effects may vary.

The enhanced survival of *Salmonella* in orange juice supplemented with TCP may have been due to a higher pH compared to that of the control (Table 2). 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, by far the largest increase effected by a calcium supplement. Tricalcium phosphate, $Ca_{10}(OH)_2(PO_4)_6$-$XH_2O$ (X is undetermined), contributes hydroxide ions that increase the pH of the juice. Similarly, the addition of calcium citrate increased the pH of the orange juice to 4.19. This

increase in pH could be due to pH buffering between citric acid in the juice and its conjugate base, citrate. This would decrease the free cation concentration, which would, in turn, increase the pH. The higher pH of the juice incurred less acid stress to salmonellae. This corresponds to the observations of others that have investigated the survival of *Salmonella* inoculated in orange juice (19). Although salmonellae in that study were acid-adapted before inoculation, the length of survival positively correlates to increased pH. Salmonellae survived for 27 days at pH 3.5, compared to 73 days at pH 4.4. This supports the expected behavior of *Salmonella* in that increased pH decreases acid stress and rate of inactivation. In our study, however, the pH of CaL-supplemented and the control juices was the same, although inactivation was much more rapid in the presence of CaL.

Another phosphate salt, trisodium phosphate, was used effectively at concentrations of 10 and 15% in 15-s dips to reduce *S.* Montevideo counts from 4.07 $\log_{10}$ cfu/$cm^2$ to undetectable populations on the surface of tomatoes (26). Compared to lactate salts, the antimicrobial activity of phosphate salts is less pronounced.

Although the pH of the orange juice was increased by adding CCM, survival of *Salmonella* was only enhanced when the inoculum was Inoculum 3. The higher number of cells in Inoculum 3 that survived in orange juice could be due to the presence of one or more serotypes with exceptional acid tolerance. The produce isolates in Inoculum 3 may be more suited to the citric acid / citrate conditions created by addition of CCM to the juice compared to serotypes in the other inocula. The addition of citrate, in addition to creating a potential buffer system, would increase the citric acid content in the juice. The sufficiently low pH (4.19) of CC-supplemented juice would have allowed conversion of some of the added citrate ion to citric acid, with $pK_1$, $pK_2$, and $pK_3$ values of 3.14, 4.77 and 6.38 (5). This may have hindered survival of the *Salmonella* in Inocula 1 and 2, but the produce isolates in Inoculum 3 could have been relatively unaffected, possibly due to a higher tolerance to acidic environments. This suggests that under specific environmental conditions, the source of *Salmonella* could play a role in its survival in orange juice.

No growth was observed on TSA containing CaL or CaL / TCP at pH 4 (Table 1); however, growth was observed on the TSA control TSA (no calcium) at pH 4. This indicates that growth of *Salmonella* is not inhibited solely by adjusting the medium to pH 4. Calcium lactate played a role in inhibiting *Salmonella* growth. Since TCP alone did not inhibit *Salmonella*, the inhibitory effect can be attributed to CaL. This is an agreement with

observations on the survival of *Salmonella* in orange juice supplemented with CaL and CaL/TCP. However, CaL in TSA at pH 7 did not cause inhibition. This does not support previous findings by others who state that some organic acid salts exhibit antimicrobial activity in a dissociated form at pH values well above their $pK_a$ (7). Strains in Inoculum 2 may be more affected by calcium ions at pH 7 than strains in Inocula 1 and 3 as evidenced by higher counts on TSA containing all calcium salts except TCP. This could be due to the effect of calcium on various cellular functions. Inoculum 3 counts in TSA containing CC and CCM treatments were higher than that of the control at pH 4, possibly due to the citrate buffering effect discussed above.

Calcium ions have been shown to stabilize pressure sensitive cellular targets in *E. coli*, increasing the pressure resistance of the cell (11). A similar mechanism could occur in *Salmonella* in which protein complexes responsible for ion transport out of cells are stabilized at low pH. The lipopolysaccharide layer of *S.* Typhimurium contains high affinity binding sites for calcium, possibly allowing calcium to play a role in structural stabilization (23). Calcium has also been thought to stabilize DNA-protein interactions. The addition of EDTA, a calcium chelator, to *E. coli* has been shown to induce premature cell division (23). All of these factors could have potentially contributed to increased survival of the *Salmonella* on calcium supplemented TSA.

In summary, the addition of calcium salts to orange juice can significantly enhance or reduce the survival of *Salmonella*. Calcium lactate was the most effective of the supplements in inactivating *Salmonella* spp. The use of calcium lactate to supplement commercial orange juice may simultaneously enhance nutritional value to consumers and reduce the viability of *Salmonella* during storage. The role of calcium lactate in a combination of physical or other chemical interventions to minimize safety risks that might be associated with unpasteurized juices should be investigated. Further investigation into the effect of calcium-supplemented juices on survival and growth of pathogens and spoilage microorganisms may provide preservation technologies that would benefit the fruit juice industry and consumers alike.

## ACKNOWLEDGMENTS

We would like to thank Mr. Jerry Davis for providing statistical analysis and support, and Dr. Ying Mao, Dr. Mahbub Islam, Ms. Joy Adams, Mrs. Andrea Burnett, Mrs. Kim Hortz, and Mr. Bob Flewellen for technical assistance.

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TABLE 1. *Salmonella* counts recovered on TSA (pH 4.0 and pH 7.0) supplemented with calcium salts

| Salmonella inoculum | Calcium supplement[a] | Population ($log_{10}$ CFU/ml) pH 4.0 | pH 7.0 |
|---|---|---|---|
| Inoculum 1 | Control[b] | 8.82 | 8.75 |
| (S.Muenchen) | CaL | < 2[c] | 8.74 |
| | TCP | 8.80 | 8.70 |
| | CaL/TCP | < 2[c] | 8.59 |
| | CC | 8.91[d] | 8.82 |
| | CCM | 8.89 | 8.80 |
| Inoculum 2 | Control[b] | 8.94 | 8.78 |
| (Human/Animal strains) | CaL | < 2[c] | 8.89[d] |
| | TCP | 8.87 | 8.81 |
| | CaL/TCP | < 2[c] | 8.89[d] |
| | CC | 8.86 | 8.85[d] |
| | CCM | 8.91 | 8.89[d] |
| Inoculum 3 | Control[b] | 8.90 | 8.84 |
| (Produce-associated | CaL | < 2[c] | 8.89 |
| outbreak strains) | TCP | 8.84[c] | 8.77 |
| | CaL/TCP | < 2[c] | 8.84 |
| | CC | 8.97[d] | 8.86 |
| | CCM | 9.03[d] | 8.92 |

[a] Control – no calcium added CaL- Calcium lactate; TCP - tricalcium phosphate; CaL/TCP- calcium lactate/tricalcium phosphate; CC- calcium citrate;

[b] There was a separate control (no calcium) for each Inoculum at each pH level

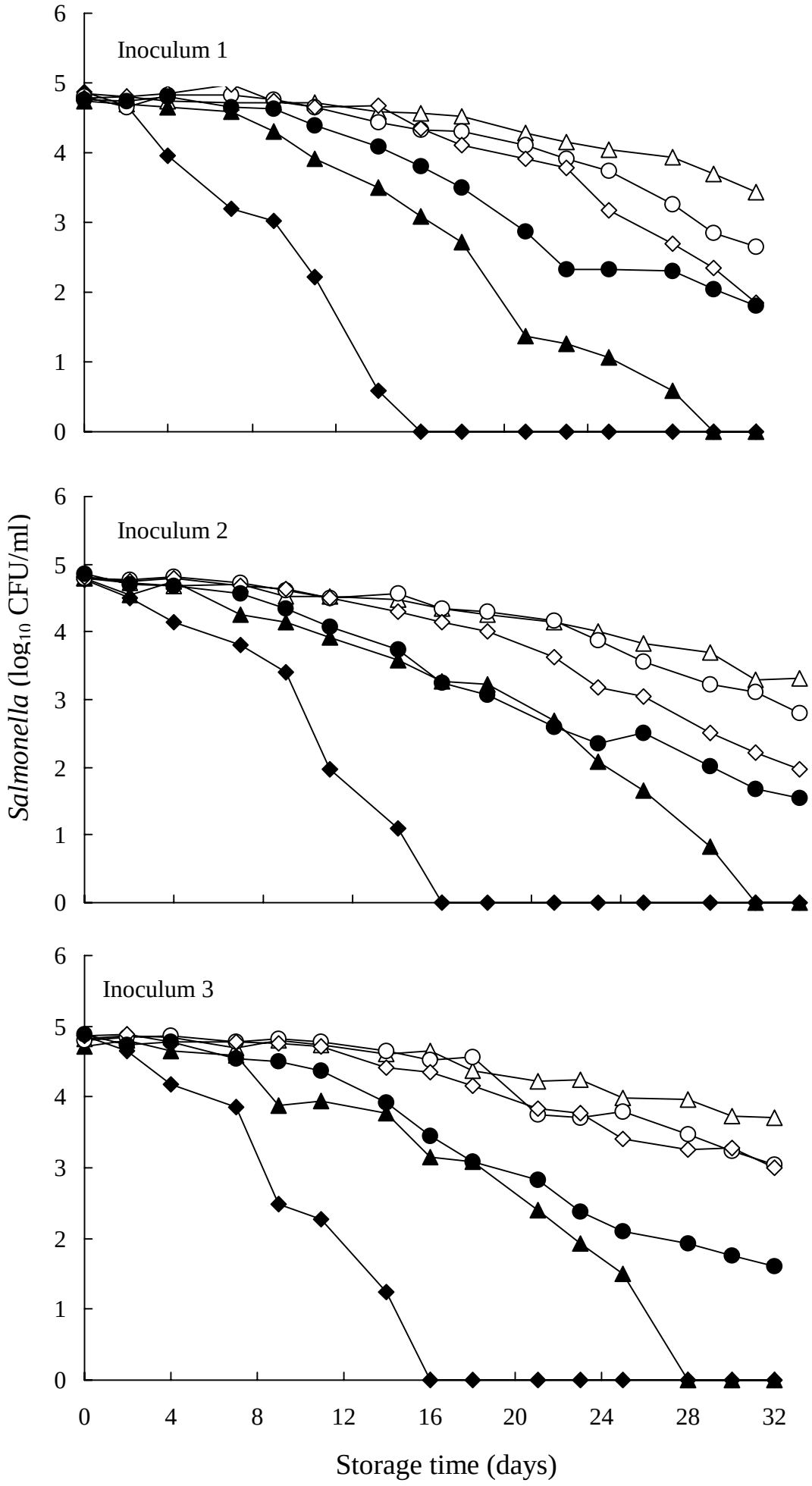
[c] Within each inoculum and pH, value is significantly lower ($p \leq 0.05$) than that of control

[d] Within each inoculum and pH, value is significantly higher ($p \leq 0.05$) than that of control

TABLE 2. Initial pH of calcium-supplemented orange juice

| Orange juice formulation | pH |
|---|---|
| Orange juice base | 3.96 |
| Calcium lactate (CaL) | 3.96 |
| Tricalcium Phosphate (TCP) | 4.29 |
| Calcium lactate/Tricalcium Phosphate (CaL/TCP) | 4.12 |
| Calcium Citrate (CC) | 4.19 |
| Calcium citrate malate (CCM) | 4.16 |

Figure 1. Survival of *Salmonella* in Inoculum 1 (*S.* Muenchen), Inoculum 2 (human and animal isolates), and Inoculum 3 (produce isolates) inoculated into orange juice supplemented with calcium and stored at 4$^{o}$C for up to 32 days. Key: juice supplemented with calcium lactate (CaL) (◆); calcium lactate / tricalcium phosphate (CaL/TCP) (▲); calcium citrate malate (CCM) (◊); calcium citrate (CC) (O); tricalcium phosphate (TCP) (Δ); and control (no calcium) (●)


Inoculum 1
Inoculum 2
Inoculum 3
Salmonella (log10 CFU/ml)
Storage time (days)
0
1
2
3
4
5
6
0
4
8
12
16
20
24
28
32

# CHAPTER 4

# SUMMARY AND CONCLUSIONS

The objectives of the research documented in this thesis were:

1. To determine if calcium supplementation affected the survival of *Salmonella* in orange juice stored at 4$^{o}$C for 32 days.
2. To determine if the source of isolation of *Salmonella* serotypes influences survival characteristics when inoculated in calcium supplemented and non-supplemented orange juice stored at refrigeration temperatures.

This research showed that the form calcium supplement used to supplement orange juice does play a role in influencing *Salmonella* survival. In orange juice fortified with calcium lactate, *Salmonella* Muenchen counts decreased more rapidly than those in non-fortified juice. Counts of *S.* Muenchen decreased more slowly in juices fortified with calcium citrate and tricalcium phosphate than those in non-fortified juices. Populations of salmonellae from human and animal sources decreased more slowly in juice fortified with tricalcium phosphate than in the non-fortified juice, while counts of salmonellae associated with produce outbreaks declined more slowly in juice fortified with calcium citrate than in non-fortified juice. Through PCR fingerprinting of five *Salmonella* serotypes from humans and animals, *S.* Heidelberg was determined to be present in 63% of the samples that were analyzed on the final day (Day 32) of storage. In the five *Salmonella* serotypes associated with produce outbreaks, *S.* Baildon and *S.* Poona were present in 43 % and 35 % of colonies analyzed on the final day of storage. There were no observable trends when comparing the survival of *Salmonella* from different sources.

This first portion of this research (Chapter 2) did not allow a direct comparison of the survival *Salmonella* in orange juice fortified with different sources of calcium. In the second part of this research (Chapter 3), calcium supplements were added to orange juice and then inoculated with *Salmonella* so that direct comparisons of the survival of *Salmonella* could be made. Orange juice supplemented with calcium lactate and a combination of calcium lactate and tricalcium phosphate significantly increased the rate of inactivation of all *Salmonella* inocula when compared to those in non-supplemented orange juice over the 32-day storage period at 4$^{o}$C. However, juice supplemented with calcium citrate and tricalcium phosphate showed less rapid declines in counts of all *Salmonella inocula* when compared to those in non-supplemented juice. Serotypes from produce-associated outbreaks showed less inactivation than *S.* Muenchen or serotypes from human or animal origin in juice supplemented with calcium citrate malate. Calcium lactate and a combination of calcium lactate/tricalcium phosphate added

to tryptic soy agar also reduced counts of all *Salmonella* inocula when compared to other calcium supplements.

The form of calcium used to fortify orange juice does impact the survival of *Salmonella*, but the source of isolation of *Salmonella* serotypes does not seem to impact their survival in orange juice. This research revealed that calcium lactate may be used as an effective antimicrobial in orange juice against *Salmonella*, while tricalcium phosphate and calcium citrate may enhance the survivability of *Salmonella* in orange juice. Further investigation of the effects of calcium supplements used in the fortification of orange juice on spoilage and pathogenic microorganisms would beneficial to the fruit juice industry and consumers alike.