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Influence of long-term survival on resistance of Salmonella enterica serovar typhimurium and Escherichia coli O157:H7 to physical or chemical food processes

Fei Wang
Iowa State University

Influence of long-term survival on resistance of *Salmonella enterica* serovar typhimurium and *Escherichia coli* O157:H7 to physical or chemical food processes

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Fei Wang

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Program of Study Committee:
Aubrey Mendonca, Major Professor
Byron Brehm-Stecher
James Dickson
Angela Shaw
Alan Dispirito

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Under adverse conditions in the natural environment or in food processing facilities bacteria may enter a long-term-survival (LTS) phase and maintain a stable concentration of viable cells for months or years. In this LTS phase, pathogens may become resistant to antimicrobial processes that would typically inactivate them in the exponential- or stationary phase of their life cycle. In this regard, the resistance of LTS phase *Salmonella enterica* serovar Typhimurium, *Salmonella* PT 30 and *Escherichia coli* O157:H7 to ultraviolet (UV) radiation, desiccation or cinnamaldehyde in model systems and in food products was investigated. Exponential-, stationary, and LTS phase cells were cultured in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; 35°C) for 4 h, 24 h and 14 days, respectively. In the UV resistance experiment, cells from each physiological state were exposed to UV light in saline (80 $\mu W/cm^2$) and apple juice (820 $\mu W/cm^2$). The LTS *S.* Typhimurium consistently exhibited the highest UV resistance. In both saline and apple juice, D-values of LTS *S.* Typhimurium were significantly higher than D-values of the other two physiological states of cells. The LTS cells also had the least sub-lethal injury in the surviving population. Both *S.* Typhimurium and *S.* PT30 in the LTS phase were the most resistant to desiccation on paper discs and on raw almonds, and to hot air drying of almonds at 100°C. The LTS *E. coli* O157:H7 exhibited the highest resistance to cinnamaldehyde in carrot juice and apple juice compared to stationary phase cells. These results indicate that the LTS state cross-protects *S. enterica* and *E. coli* O157:H7 against UV radiation, desiccation and cinnamaldehyde. Additionally, we demonstrated that LTS cells are highly tolerant to antibiotics compared

to stationary phase cells and that inhibition of protein synthesis results in the death of those cells.

CHAPTER 1. GENERAL INTRODUCTION

Outbreaks of illness caused by bacteria and parasites have been associated with juices [1-3] and dry food [4-7] for decades. From 1923 to 2000, contaminated fruit juices have been implicated in at least 28 foodborne illness outbreaks [8]. Enteric foodborne pathogens such as *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium, parasitic protozoa including *Cryptosporidium parvum*, and Gram-positive pathogens like *Listeria monocytogenes* have all been reported to survive in raw fruit and vegetable juices [9-12]. Although *Salmonella* does not grow in low-water activity (low-a_w) foods, the long-term survival of this organism in chocolate, dried eggs, hard cheese, almonds, salami, and infant dried milk has been well documented [13].

Several traditional microbiology textbooks describe the bacterial life cycle as consisting of three or four phases[14]. However, there are actually five phases namely, lag phase, exponential phase, stationary phase, death phase and extended or long-term stationary phase [14, 15], which is the period of prolonged survival [15]. Long-term-survival (LTS) phase has been found in *Listeria monocytogenes* [16], *Escherichia coli* [14], *Micrococcus luteus* [15] and another microorganism [17], in which viable cell densities are kept stable. An increasing number of pathogens such as *L. monocytogenes* persist in food processing plants for a long time and are resistant to environmental stresses. This issue poses a significant threat to both public health and the food industry since those pathogens can cause life-threatening diseases [18, 19]. Entry into a LTS phase may account for its long-term persistence in food processing plants [16]. In the LTS phase, *L. monocytogenes* cells become coccoid-shaped, more resistant to heat and high pressure, and maintain a stable population at ~10^8 CFU/ml for at least 1 month [16].

Aging broth cultures of *Sarcina lutea* and *Serratia marcescens* entered a "senescent phase" and remained alive during a two-year incubation period [15]. *E. coli* can be maintained for a long period of time without the supply of nutrients[15, 20, 21] in what is called a “long-term stationary phase”[14]. Also, bacteria in the natural environment are known to endure starvation and other adverse conditions in which they survive for very long periods of time until nutrients and favorable conditions are available. However, published reports describing the LTS phase in *S.* Typhimurium and *E. coli* O157:H7 in terms of their resistance to food processing or preservation methods are non-existent.

Accordingly, the first objective of this research was to evaluate the extent of resistance of LTS cells of *S.* Typhimurium to UV radiation in saline and apple juice. The second objective was to determine the resistance of LTS cells of *S.* Typhimurium and *S.* PT 30 to desiccation on paper disc and almonds. The third objective was to determine whether LTS cells of *E. coli* O157: H7 were more resistant to cinnamaldehyde than stationary phase cells in carrot juice and apple juice. The fourth objective was to determine if, like “persister” cells, LTS cells exhibited antibiotic tolerance and a cessation of protein synthesis.

Dissertation Organization

This dissertation is divided into seven chapters. The first chapter is comprised of a general introduction. The second chapter is a literature review containing information related to the research conducted in chapters 3 through 6. The final chapter provides a general summary of the research performed. All pertinent figures, tables, and graphs appear at the end of their respective chapters, which all follow a specified journal format. At the conclusion of each chapter references can be found formatted for journal specifications. It is the intent that Chapter 3, 4, 5 and 6 be submitted to Foodborne

Pathogens and disease. The abstract titled “Ultraviolet radiation resistance and injury of long-term-survival phase cells of *Salmonella* Typhimurium ATCC 14028 in 0.85% saline and apple juice” was presented at the annual meeting of the International Association of Food Protection in Portland Oregon (July 2015). The abstract titled “Desiccation resistance of different phases cells of *Salmonella* Typhimurium and *Salmonella* PT 30 on paper disc and almonds” was presented at the annual meeting of the International Association of Food Protection in Tampa Florida (July 2017).

CHAPTER 2. LITERATURE REVIEW

Long-term survival cells

Five phases of the bacterial life cycle

The bacterial life cycle is commonly represented by four phases namely lag phase, exponential phase stationary phase and death phase. However, a fifth phase called extended or long-term phase has been reported [14]. This fifth phase has been referred to as the "senescent phase" in *Serratia* and *Sarcinia* spp or "long-term stationary phase" in *Escherichia coli* [14]. Bacteria in the natural environment may enter a starved state from being deprived of nutrients and survive for long time until the relief of starvation [17]. After the death phase, *E. coli* can be retained in batch culture for a very long time without added nutrients [15, 20]. Via constant addition of sterile distilled water to retain the volume and osmolality, aerobically grown cultures can be maintained in a viable state at densities of ~10^6 colony forming units (CFU)/mL for more than five years without the supply of nutrients This observed behavior in bacteria is representative of the long term survival phase which is different from the commonly observed early and full stationary phase of the bacterial life cycle. Distinct from early stationary phase, in which little cell division is present, full stationary phase is a dynamic phase represented by balanced 'birth' and 'death' rates [14]. Actually, long-term batch cultures of stationary phase cells have an obvious carrying capacity, which can only support a limited number of cells. Therefore, other cells must die while new cells are generated [22].

Persister cells

In nature, most bacteria are present in a dormant state where they have to survive inconsistent periods of altering environmental stresses [23]. Since the initial discovery of

persister cells [24], those type of bacterial cells have been defined as a small and rare subpopulation of phenotypic variants tolerant to many antibiotics [25, 26]. Persister cells can tolerate antibiotic treatments because of their dormant nature, but they "awaken" and start to multiply upon the removal of antibiotics, which explains the fact that they are responsible for quite a few recurrent and chronic infections [25-27]. High persister (hip) mutants of *Pseudomonas aeruginosa* have been isolated in patients with cystic fibrosis. Likewise, hip mutants of *Candida albicans* have been isolated in patients who had an oral thrush biofilm. The abovementioned observations clearly imply that persisters may be the major culprit leading to the recalcitrance of chronic infectious disease to antimicrobial therapy [26].

Infectious disease is sometimes "untreatable", even when caused by a pathogen, which is not resistant to antibiotics, and this is the vital paradox of chronic infections. Mostly, chronic infections are associated with the formation of biofilms, which is likely a major contributor to the problem [28, 29]. Biofilms cannot fully prevent penetration of antibiotics [30], but mainly they generate a barrier for the larger components in the immune system [31-33]. More importantly there could be non-specific binding of antibiotics to components of the biofilms thus reducing the antibiotic concentration available for the target pathogen. Also, target cells at the bottom of the biofilm, depending on the thickness of the biofilm, my experience a lower concentration of antibiotic compared to cells at the upper parts of the biofilm. The presence of biofilm-specific resistance mechanisms was proposed to explain the recalcitrance of infectious diseases [34]. Nevertheless, the bulk of cells in the biofilm are greatly susceptible to be killed by antibiotics and only a small fraction of cells named persisters remain alive [35].

According to these findings, a simplified model of the relapsing chronic infection has been proposed: Antibiotics eliminates most cells, and the immune system kills regular cells and persisters from the bloodstream [36]. It seems the only surviving cells in the biofilm are persisters which repopulate the biofilm and subsequently cause relapses in infection once the level of antibiotic decreases. Apparently, biofilms function as a protective habitat for persisters [35, 37-40], and enable them to evade the immune response. Nevertheless, a more general paradigm is that persisters are crucial for pathogens to survive antimicrobial chemotherapy whenever the immune response is restricted. It is likely that persisters play an important role in immunocompetent individuals in cases where the pathogen is poorly accessed by the immune system's inability to penetrate biofilms. It has been reported that all free-living bacteria can form persister cells, yet it is challenging to study those cells because of to their seemingly rare frequency within a population [26].

To date, all persister cell research has been done with cells in the exponential-to-stationary phases [25, 41, 42], but not with the long-term-survival phase (LTS) cells. The LTS phase is achieved after extended incubation following a death phase in which 90-95% of the population is eliminated [16, 22], largely owing to production of an extracellular death factor at high density of cells [43, 44]. Previous studies have reported persister cells with low frequency in the exponential phase [42, 45], in which all cells are metabolically active. Most studies have revealed that persister cell frequency increases significantly beyond the exponential phase and comes to a maximum of ca. 1% in the stationary phase. To determine how persister cells form in the exponential phase without a stringent response, many researchers have proposed such concepts as stochastic

fluctuation in persister proteins, micro-starvation, bistability, and/or bet-hedging [25, 46, 47]. In contrary, several reports have revealed that the stringent response and toxin-antitoxins are general in free-living bacteria and play an important role in the formation of persister cells [26, 27, 42, 47] and endospores [48, 49]. Currently, the majority of bacteria are classified as "non-spore forming", whereas only a few are categorized as forming endospores which are metabolically inactive and very resistant to numerous stresses. Spores are not unique to bacteria because they can be found in other living organisms including molds, yeast and mushrooms, however, endospores are unique to bacteria but are not the exclusive type of spores found in this microbial group. A different type of spores called exospores are formed by the non-endospore forming genera *Myxococcus* and *Streptomyces* [50, 51]. Therefore, a lack endospore formation should not serve as a criterion to conclude that most bacteria are "non-spore formers". While much is currently known about bacteria classified as spore formers, it is still unclear how persister cells form in "non-spore forming" bacteria and what is their true biological role [25]. More recently, studies using the foodborne pathogen, *Listeria monocytogens*, have attempted to clarify this important issue [16].

Listeria monocytogenes shrinks from a rod to a coccoid form after it goes to the LTS phase like many other "non-spore forming" bacteria [16]. LTS phase cells can be alive in trypticase soy broth with added yeast extract (TSBYE) for more than one year at a constant cell density of about log 8.4 CFU/ml, but "germinate" fast and finish their life cycle in fresh TSBYE [16]. Similar to several previous reports with other "non-spore forming" bacteria, *L. monocytogenes* persister cells occur at a low frequency in the stationary phase. Nevertheless, contrary to previous reports in which only exponential-to-

stationary phase cells were used, LTS phase cells that were over one month old and treated with ofloxacin or penicillin were not eliminated [24]. Those results indicate at least a 100-fold enhancement in persister cell frequency, compared to all previous reports, which have demonstrated a maximum persister cell frequency of 1% in the stationary phase [25]. Those findings also imply that persister cells are not rare phenotypic variants, but can be a 100% of the population in the LTS phase. Additionally, , previous reports indicated that synthesis of extremely low levels of "survival proteins" is needed for long-term viability in the stationary phase [23, 52]. Therefore, inhibition of the production of long-term-survival proteins might be the long-sought "Achilles Heel" of persister cells. Antibiotics that target and inhibit their synthesis [41] may significantly reduce or even eliminate various chronic and recurrent infections, which hold great promise in clinical applications.

Characteristics of LTS phase cells

The LTS phase cells of *L. monocytogenes* are dramatically more tolerant to harsh conditions such as heat and high pressure than cells in stationary phase [16]. Novitsky and Morita et al. [53] demonstrated that one week of starvation enhanced the barotolerance of a marine vibrio, which also altered cellular morphology from rods to cocci [54]. The boosted barotolerance in the LTS phase is presumably due to cytoplasmic condensation and subsequent lowering of water activity in coccoid cells. Low cellular water activity was previously shown to strongly enhance the barotolerance of *L. monocytogenes* [55]. Starvation boosts heat resistance in various microorganisms, such as *L. monocytogenes* [56, 57], *E. coli* [58], *Arthrobacter globiformis* [59], and *Lactococcus lactis* [60]. It is likely that the high thermotolerance in the LTS phase in *L. monocytogenes* is due to a lowered water activity in the coccus-shaped cells, which is

similar to what is expected to occur in the core of bacterial spores [61-63]. It is a reasonable assumption because non-spore-forming cells can benefit from a fitness strategy by producing LTS sells in natural environments. It is also likely that starvation stress before or during the LTS phase brings about the synthesis of stress proteins, which would protect cells against heat [58] and pressure [64].

The set of all mRNA molecules (transcriptome) of *L. monocytogenes* changes significantly as that pathogen transits through different phases of its life cycle and ends in a very unique transcriptome in the LTS phase, in which genes associated with transport of the compatible solute trehalose is greatly up-regulated [65]. In a population of Listeria cells that went into death phase, the majority of cells were dead, while appropriately 10% of the population survived. Fourteen genes related to protein synthesis were up-regulated in death phase including ribosomal protein genes and a gene encoding a translation initiation factor. Newly synthesized proteins at the early stage of starvation were important for maintaining the LTS phase of *L. monocytogenes* [56, 66] and *E. coli* [67]. Surviving cells could live or even grow on nutrients from dead cells and such phenomenon was defined as "cryptic growth" [22]. The death of the majority is likely a fitness strategy to maintain survivors for reproduction in the future [68]. The surviving population degraded rRNA from dead cells to gain additional nucleotides and energy [69] to support their metabolism during the following LTS phase. The transcriptional profiles at LTS phase revealed up-regulation of three genes encoding transporters for compatible solutes including glycine betaine and trehalose [65]. In the LTS phase, high levels of compatible solutes might be taken up from the growth medium and accumulated in the cytoplasm, leading to the increased thermo- and barotolerance. Glycine betaine can be

accumulated in cells of *L. monocytogenes* under osmotic or salt stresses thereby increasing osmotolerance or cryotolerance [70, 71]. Likewise, accumulation of trehalose in bacteria could be induced by various adverse conditions to protect cells against stresses such as cold, heat, desiccation, and oxidation [72]. It was acknowledged that high concentrations of trehalose result in lowered water activity [73] and that condition promotes barotolerance in *L. monocytogenes*, possibly via stabilizing proteins [55].

LTS-phase population dynamics

Stressful environmental conditions for bacteria can select for mutants that express the growth advantage in stationary phase (GASP) phenotype [14]. GASP is defined by the capacity of cells aged in long-term batch cultures to outcompete cells in younger cultures. The appearance of the GASP phenotype serves as the signal phenotype linked to changes observed in long-term batch cultures [20, 74-76]. The discovery of GASP and relevant studies on its genetic basis have been reported previously [74, 76]. By far, there has been four GASP mutations identified in *E. coli* and three of the loci are thoroughly characterized [75-79]. One identified GASP mutation is in *rpoS* [75] which encodes the alternative sigma (σ) factor RpoS or σ^S. After the occurrence of GASP alleles of *rpoS*, novel GASP mutations continue to appear upon incubation of the bacterial cells and this has been validated in several ways. First, cells from 10-day-old cultures outcompete 1-day-old cells. Moreover, 20-day-old cells outcompete 10-day-old cells, and 30-day-old cells outcompete 20-day-old cells, and etc. [20]. In fact, cells in cultures aged up to 60 days outcompete cells from any younger cultures with different degrees of relative fitness [80]. For instance, 10- to 20-day-old cultures always display a strong GASP phenotype and drive majority of younger cells to extinction. Cells from 30- to 60-day-old cultures

always exhibit the GASP phenotype, but they coexist with formerly majority cells and drive them to extinction. This continuous appearance of GASP mutations over time demonstrates that LTS cultures are not static, but highly dynamic. After about 60 days of incubation, more mutants that intend to develop the GASP phenotype but fail are found, as well as the mutant possess no increased competitive ability [81]. Those phenomenon imply that the culture environment after 2 months of incubation is adequately distinct from overnight cultures to prevent aged cells from expressing a fitness advantage over 1-day-old, unaged cells [14].

Salmonella

Salmonella spp. is anaerobic gram negative, rod-shaped bacteria from the family of *Enterobacteriaceae*[82]. *Salmonella* contains two species, namely, *Salmonella bongori* and *Salmonella enterica* (*S. enterica*). There are six subspecies of *Salmonella enterica* including *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI) [82]. To date, more than 2500 serovars of *Salmonella enterica* have been identified and the serovars of the subspecies are classified according to carbohydrate flagellar and lipopolysaccharide structures [83].

Sources of contamination

Salmonella is widespread in the natural environment and its existence is largely due to a wide variety of animal reservoirs of this organism. *Salmonella* is able to colonize the intestinal tracts of vertebrates, such as humans, wildlife, domestic pets, and livestock [82]. The only serotypes of *Salmonella* found in humans are *Salmonella* Paratyphi and *Salmonella* Typhi [84]. Typically, *Salmonella* foodborne disease outbreaks are linked to consumption of contaminated foods such as eggs, poultry, dairy products, and certain dry

food products such as powdered milk, coconut, chocolate, raw almonds, toasted oats breakfast cereal, and dry seasonings. Also, this pathogen is harbored by snakes, frogs, iguanas, pet tortoises, small turtles, aquatic turtles, pet crested geckos, and pet bearded dragons [85-88]. Recently, an increasing number of incidents of fruits and vegetables like broccoli, cilantro, cauliflower, and spinach as transmission sources of *Salmonella* have been reported [86, 89]. Fresh product contamination is largely due to *Salmonella* entering the food through natural uptake, scar tissues, through root systems and transfer to edible plant tissues when slicing produce.

Growing incidents of *Salmonella* contamination in fresh produce are possibly due to the increased importation of fresh produce from foreign countries. Fresh produce could be contaminated during production, collecting and processing. Many tropical countries have climates offering suitable temperature for the growth of fresh fruits and vegetables during the whole year. Furthermore, Good Manufacturing Practices (GMPs) and Good Agricultural Practices (GAPs) in several countries during production, collecting, packaging, and distribution of fruits and vegetables may not fulfill the minimum standards established by the United States (U.S.). Hygienic conditions below minimum standards are an important factor microbial contamination of fresh produce. Although poor hygienic conditions play a critical role in microbial contamination, it is not the only reason. Other factors like washing fresh produce with contaminated water, fertilizing crops using untreated sewage water, and inappropriate handling of produce, all facilitate the persistence of *Salmonella* in fresh vegetables and fruits [90].

Salmonella is able to propagate under a variety of environmental conditions. For example, *Salmonella* could multiply in the presence of 0.4 to 4% sodium chloride and

grow between 5 to 47°C with an optimum growth temperature range of 35 to 37°C. Given optimal intrinsic (pH, water activity, re-dox potential) and extrinsic factors (atmosphere), some serotypes of *Salmonella* can even multiply slowly at temperature as low as 2 to 4°C or high as 54°C. At water activity between 0.99 and 0.94, *Salmonella* is able to multiply. In dried foods, *Salmonella* could survive in a dried state at a water activity of <0.2 whereas this organism can lose viability at pH<3.8, water activity <0.94 or at temperatures <7 ºC [91].

Salmonellosis

Salmonella enterica is responsible for ca. 1.1 million non-typhodial illnesses, 19,000 hospitalizations, and 378 deaths in the U.S. annually [92]. One of the most prevalent serotypes of *Salmonella* in developed countries is *Salmonella enterica* serotype Enteritidis. *Salmonella* Enteritidis, was a main cause of foodborne human illiness in the 1980s [93]. According to the Centers for Disease Controls and Prevention's "Estimates for Foodborne Illness" [94], *Salmonella* was rated as one of the most problematic pathogens based on the number of related illnesses, hospitalizations, and deaths. On the basis of that assessment, *Salmonella* was responsible for approximately 1,207,561 illnesses, 19,336 hospitalizations, and 378 deaths. Raw or undercooked poultry and eggs are typically associated with *Salmonella* infections. Thant trend is starting to change slowly as other types of food products are implicated in salmonellosis foodborne outbreaks. In the past decade, *Salmonella* infections have been attributed to consumption of fresh produce and fully processed foods. For instance from 2006 to 2015, various serovars of *Salmonella* were responsible for foodborne infections involving contaminated food products including cucumbers, nut butter, bean sprouts, chia powder, sesame paste, raw cashew cheese, ground beef, peanut butter, cereals, tomatoes, pistachios, pot pies

cantaloupes, alfalfa sprouts, red and black pepper, papayas, frozen fruit pulp, and ground tuna product [95]. While many of these cases caused only a few *Salmonella* infections, there have been some cases with a large numbers of infected persons. In 2007, *Salmonella* serotype Tennessee was linked to a multistate outbreak in involving peanut butter, which led to 425 infections from 44 states, 71 hospitalizations and 0 deaths [96]. In the same year, *Salmonella* I 4,[5],12:i:-("four five tewlve eye minus"), in pot pies, was responsible for 272 infections and 65 hospitalizations [97]. In 2008, jalapeno peppers, tomatoes and serrano peppers, contaminated with *Salmonella* Saintpaul, were responsible to cause 1442 infections, 286 hospitalizations, and 2 deaths [98]. In 2009, *Salmonella* Saintpaul, present in raw alfalfa sprouts, caused 235 infections in 14 states, 7 hospitalizations and zero deaths [99]. Another notable outbreak in the same year was ascribed to *Salmonella* Typhimurium. *Salmonella* Typhimurium in Peanut butter was caused 714 infections in persons from 46 states, 171 hospitalizations, and 9 deaths [100]. In 2010, *Salmonella* Montevideo in peppers/Italian-style meats caused 272 infections, 52 hospitalizations, and 0 deaths [101]. In that same year, *Salmonella* Enteritidis was associated with contaminated eggs in Iowa resulting in about 2000 illnesses in 11 states [102]. In 2012, both *Salmonella* Typhimurium and *Salmonella* Newport, in cantaloupes, caused 261 illnesses, 94 hospitalizations, and 3 deaths [103]. Additionally, raw scraped ground tuna with *Salmonella* Bareilly and *Salmonella* Nchanga was linked to 425 illnesses, 55 hospitalizations [104]. Recently, cucumbers were a source of *Salmonella* infections. In 2014, *Salmonella* Newport in cucumbers were responsible for 275 illnesses, 48 hospitalizations, and 1 death [105]. Also, cucumbers, contaminated with *Salmonella* Poona was again responsible for 732 illnesses, 150 hospitalizations, and 4 deaths [106].

These previously stated salmonellosis foodborne outbreaks underscore the fact that the widespread occurrence of *Salmonella* in the natural environment due to numerous animal reservoirs increases its chances of contaminating a variety of food products.

Characteristics of salmonellosis

Salmonellosis is known as the infection caused by *Salmonella* that can result in disease symptoms like gastroenteritis, typhoid fever, bacteremia, and focal (localized) infections in extra intestinal parts of the body [107]. Gastroenteritis, as a result of *Salmonella* infection typically subsides after several days, but more severe cases especially those involving immunocompromised individuals or individuals with systemic infections may be subject to antibiotic therapy [108, 109]. The people most susceptible to salmonellosis are the infants, elderly, and weak-immune individuals. Upon infection with *Salmonella*, most people develop vomiting, diarrhea, and abdominal cramps 12 to 72 hours after eating the contaminated food product [95]. Moreover, reactive arthritis (also known as Reiters syndrome) can develop after *Salmonella* infections in certain individuals [110]. The Reiters syndrome can last from several months to years and usually lead to swelling of feet, knees and ankles [111]. Treatments for *Salmonella* infections require taking fluids orally and the disease can be resolved within 5 to 7 days. If the infection spreads from the intestines to other parts of the body, antibiotics such as ampicillin, trimethoprim-sulfamethoxazole, or ciprofloxacin are needed [95]. While antibiotics have been proved effective in eliminating *Salmonella*, some species have exhibited antibiotic resistance as a result of being harbored in animals fed antibiotics to faciliate growth. The cost of damages due to outbreaks of *Salmonella* in the U.S. were estimated to be between $600 million and $3.6 billion dollars per year [112].

Considering the extent of human suffering from salmonellosis and the high economic burden posed by this disease it is imperative that effective intervention kill steps be implemented to destroy salmonellae in food products while meeting current consumer demands for nutritious, minimally processed foods with fresh or fresh-like characteristics.

UV light for inactivation of foodborne pathogens

Nowadays consumers are demanding tasty, healthy, safe, natural foods produced in an environmentally friendly manner. With the increasing negative public concern over foods containing synthetic chemical preservatives, UV light processing has emerged as a popular non-thermal alternative food preservation treatment with positive consumer feedback. Although the utilization of UV light is well established in water treatment, air disinfection and surface decontamination, its use is still limited in food treatment [113]. UV light is highly effective in treating transparent liquid foods like clarified juices and soft drinks, but less effective in treating turbid liquid with particulates, e.g., orange juice, where UV light is strongly scattered or reflected [114]. The U.S. Food and Drug Administration (U.S. FDA) and U.S. Department of Agriculture (USDA) concluded that the use of UV irradiation in food processing is safe. In 2000, the FDA approved the use of UV light as treatment to thermal pasteurization of fresh juice products [115]. Furthermore, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) revised the definition of "pasteurization" for foods. It includes any process, treatment, or combination that is applied to food to destroy the most pertinent microorganism(s) of public health significance [114].

As a starting point to understand UV light technology, previous studies are provided on the source of UV light and mechanism of microbial inactivation by UV light.

The applications of UV light on food processing are also briefly discussed in this review, including pasteurization of liquid food and beverages, postlethality treatment for solid food and treatment of food-contact surface.

Source and mechanism of UV light

The source of UV light can be divided into two parts including solar radiation and artificial source. The sun emits radiation across a wide range of wavelengths, but the relative intensity of ultraviolet radiation reaching the earth's surface depends on attenuation by the atmosphere through absorption and scattering. Most UV for food treatments are from artificial commercial UV source, including short, medium and long wave UV lamps [116]. Short-wave UV lamps produce radiation ranging from 250 nm to 260 nm, which is lethal to most microorganisms and the peak of maximum effect appears to be at 254 nm. The success of UV technology is dependent on appropriate matching of the UV source parameters (mainly the radiating properties of the UV source) to the specific demands of the required UV application. Short-wave UV has a bactericidal effect, making it extensively useful to the food industry. It is important to know that radiation below 260 nm can produce ozone, which must be carefully monitored to prevent threat to human health.

UV light inactivates microorganisms by destroying their nucleic acid, which is either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and preventing microbial multiplication. Hydrogen bonds are formed between adjacent pairs of thymine or cytosine to form pyrimidine dimers on the same DNA strand. Dimers prevent microorganisms from replicating their DNA thus inactivating them and rendering them unable to cause infection. Nevertheless, damage to nucleic acid does not influence other functions of the cell. Repairing enzyme mechanisms have capacities to recover the cell from damage.

Such mechanisms include excision repair, photo-reactivation, recombination repair and inducible error-prone repair [117-119].

Factors related to UV efficacy

Microorganisms differ in the sensitivity to UV light due to the different cell wall structure, thickness and composition. The presence of UV-absorbing proteins and different nucleic acid structures can also influence the antimicrobial effectiveness of UV light. Previous studies demonstrated the effectiveness of UV light for inactivating *Cryptosporidium parvum* in apple cider [120]. UV light treatment caused 3.8-log reduction of *E. coli* O157:H7 in apple cider [121]. At least 5-log reduction of an *E. coli* surrogate for O157:H7 was achieved in multiple trials in treating apple cider with UV light [122, 123].

The extent of microbial inactivation by UV light is directly dependent on the UV dose applied to the food product. However, other factors including UV reactor design and the fluid dynamics parameters and absorptive properties can also affect the efficacy of UV treatment. Food products have a wide range of chemical and physical properties including pH, dissolved solids, and suspended solids, pigmented components and absorbance. In this regard it is necessary to understand how variations in certain food product characteristics can affect UV inactivation.

pH and dissolved solids

Most apple juice and orange juice have a pH between 3.3 and 4.1 [124] and enhanced acid resistance of bacteria has caused foodborne outbreaks in these acidic fruit juices. It is of great significance to explore the relationship between pH of medium and efficiency of UV treatment. Quintero-ramos et al. reported that different pH values of apple cider had no significant effect on the inactivation of *E. coli* at high UV doses [124].

Hijiner et.al [125] reported similar results related to the effect of pH the suspending medium (water) on UV resistance of bacteria, viruses and protozoan oocysts. For juices made from different apple cultivars (artificially inoculated with *E. coli* O157:H7) and treated with UV radiation. There is no statistically significant differences between log reductions of *E. coli* strains as affected by juice parameters of dissolved solids (°Brix), pH and malic acid content [126]. Furthermore, pH and °Brix, individually, had no significant difference on the rate of *E. coli* K12 destruction under the conditions tested. A small but statistically significant effect was noted when both pH and °Brix were changed and a combined effect was observed [121].

Absorbance

Absorptivity is one of food characteristics defined as the proportionality constant between food absorbance (at 254nm) and its concentration. A previous study in irrigation water demonstrated that the inactivation rates of pathogens increased with decreasing UV light absorbance [127]. The absorbance of a model solution also influenced the inactivation rate of *E. coli* K12 [121, 128], which suggests that light intensity decreases with increasing absorbance of a medium. Oteiza et al. [129]reported the efficacy of UV light inactivation of *E. coli* and *E. coli* O157:H7 in fruit juice with different absorptivity. Those same authors found a linear relation between the radiation dose and the absorptivity coefficients in all juices, indicating that the higher the absorbance of the juice, the greater the dose of UV radiation to damage *E. coli* strains.

Suspended solids

The amount of particles or suspended solids (SS) in water is an important concern in UV-treated wastewater[128]. Sometimes microorganisms can survive even under continuous exposure to high dose of UV light and SS are the key reason of this

phenomenon [130]. Particles are impenetrable to light and therefore shields microorganisms from UV light treatment [131]. The inactivation rate of *E. coli* K12 in malate buffer containing 2.5g/ml SS decreased due to the particles, compared to the buffer containing without SS [128]. In commercial apple juices, faster inactivation of *E. coli* K12 was observed in less turbid apple cider of 1400 Nephelometric Turbidity Units (NTU) compared to 2400NTU, which negatively impacted the effectiveness of UV inactivation [121].

Temperature

Temperature plays a critical role on activity of repair enzyme and configuration of nucleic acid. Inactivation rate of *E. coli*, *Candida parapsiosis* and f2 phage increased with decreasing temperature during UV processing [132]. UV dose is considered independent of temperature; the rate of repair is temperature dependent. It was concluded that the efficacy of V disinfection is not affected by temperature [125]. However, when the UV light transmittance through the liquid and reflectance at the air-liquid surface, the physical prosperities of the liquid are changed by increasing temperature, not directly by UV light [113]. The effects of temperature should be considered when comparing antimicrobial efficacies of UV treatments.

Applications in liquid food and beverage

UV light is a well-established perseveration method to reduce the microorganisms in contaminated liquid foods and beverages, which include juices, brines, liquid sugars, pharmaceuticals, processed lubricants, other semitransparent and opaque ingredients or foods. Generally, food processors would prefer to have retention of nutrients and desirable flavor characteristics in pasteurized foods while controlling pathogenic and

spoilage microorganisms. The brewing industry is a major user of UV disinfection systems to maintain the quality or taste of beer during pasteurization [133].

The physical properties of fresh juices are variable and may represent several factors, which may affect the efficiency of UV treatment. The physicochemical properties of three commercially packaged clear apple juices their absorption coefficients, soluble solids content (°Brix), pH, color (*L, a* and *b-values*) and vitamin C content are shown in table1 [114]. In those apple juices, pH and °Brix exhibited little or no effect on absorption of UV light into the juices. However, SB (brand of apple juice) juice showed the highest absorption coefficient and OS (brand of apple juice) juice was the least absorptive in terms of UV light. The juices enriched with vitamin C such as SB and OS had the higher absorption coefficients, so juices enriched with vitamin C would require higher UV doses [114]. Based on results of that study the combination of physical and nutritional properties, such as liquid density and viscosity, vitamin C content should be taken into consideration in the applying UV treatments to juices to meet the FDA's juice HACCP rule of 5-log reduction of the pertinent pathogen in juice.

The biggest disadvantage of UV processing is lack of penetration, which limits the application of this technology in opaque liquid food and beverages due to presence of dye, organic solutes and suspended matter [114]. Improved penetration of UV light into opaque liquid foods to increase the efficacy of microbial inactivation may be achieved by good reactor design. In a turbulent flow system, which pumped milk at high velocity through transparent tubes of 1cm diameter, UV radiation destroyed 99% of bacteria initially in the milk [134]. The UV processing reduces total aerobic plate count and yeast and molds in orange juice in a thin film UV reactor, which was designed and constructed

from glass. In that reactor the juice flowed along the inner surface of a vertical glass tube as a thin film [135]. However, UV treatment seems unlikely to completely replace thermal processing because it does not preserve the quality of milk [134] and degrades vitamin C in orange juice [135]. Another disadvantage to use of UV treatment in liquids is lack of monitoring or confirmation that the UV treatment has taken place. For example, heat pasteurization can be monitored by the acid phosphatase test if the treatment temperature is below 100°C.

TABLE 1. Physical Properties of Clear Apple Juices

Apple Juice	pH	Brix	Absorption coefficient,mm^{-1}	Vitamin C, mg/ml		L-values	a	B
Sahara Burst (SB)	3.49	11.9	3.91	0.30	0.12	5.74 ± 0.09	−0.667 ± 0.022	4.44 ± 0.25
Ocean Spray (OS)	3.44	11.65	0.71	0	0	4.03 ± 0.12	−0.35 ± 0.017	2.60 ± 0.04
GFS	3.51	11.75	3.71	0.45	0.22	4.67 ± 0.42	−0.39 ± 0.07	3.97 ± 0.12

Adapted from Koutchma, 2008 [114]

Applications in solid food and surface

UV light is used in the food industry for disinfecting surfaces such as those of equipment, cheese, meat and packaging containers. However, the use of UV processing is not widespread in food industry due to a limited range of commercially available equipment. Additionally, complex microbial interactions involved in microbial attachment to surface materials pose challenges to predict the extent of surface disinfection [114]. Gardner and Shana (2000) proposed a model for UV inactivation of *Bacillus subtilits* spores on filter paper that divided the paper surface into several zones based on the ratio of the UV fluency rate actually experienced by the microorganism [136]. With the increasing number of researchers working on UV treatment for solids, the application of UV processing in industry is growing quickly, including ready-to-eat

(RTE) meats, bread, fish, shell of eggs, whole and fresh-cut fruits and vegetables, and powders.

RTE meat

Microbial contamination of animal carcasses during slaughtering processing is problematic in meat industry [137]. Studies on UV light have shown that disinfection surface of meat product is feasible. UV light can be used to reduce certain pathogens, like *Escherichia coli* and *Salmonella* on pork meat surface [138], as well as *Salmonella typhimurium* on poultry carcasses [139], *Listeria monocytogenes* on chicken meat [140]. Unlike chemical or physical agents, UV light offers several advantages, since it has no residues after treatment and is not sensitive to temperature and moisture. A major disadvantage in use of UV light to decontaminate animal carcasses is its inability to reach organisms that might be embedded in fat smears, in crevices of lean tissue or in feather follicles. This problem is linked to the poor penetrating power of UV light. Additionally, shadows created by uneven surface tissue can contribute to protection of organisms from contact with UV light.

Whole and Fresh-Cut Fruits

UV radiation has been applied to whole and fresh-cut fruits to inactivate microorganisms on fruit surfaces without causing undesirable quality changes. For example, the microorganisms on packaged watermelon cubes were reduced more than 1-log by UV light without affecting juice color, leakage and overall visual quality [141]. Fresh-cut pieces of cantaloupe melon had less aerobic mesospheric and lactic acid bacteria when they were held under UV light during cutting, compared to the post-cut-treated pieces [142]. Pulsed UV (PUV) may provide effective reduction of fungal

pathogens on the surface of fresh fruits (i.e., apples, kiwi, lemon, nectarines, oranges, peaches, pears, raspberries, and grapes) [143] .

Microbial resistance to desiccation in low-water activity foods

Few organisms can tolerate physiological limitations resulting from the removal of water from cells, the storage of cells in a dehydrated state, and the rewetting of air-dried cells. To date, no group of organisms is capable of tolerating air drying, and desiccation tolerance has been evaluated in bacteria, plants (including seeds), yeasts, insects, fungi and their spores [144-146].

Dry foods are believed to pose a minimal risk for foodborne illness since their water activity is too low to allow the growth of most microorganisms. The low-water activity (low a_W) of foods is a major factor that prevents multiplication of foodborne pathogenic bacteria; therefore, foods with low a_W have often been assumed to be microbiologically safe. However, recently low-water activity, low moisture content, or dried foods, have been increasingly linked to food products recalls and foodborne outbreaks owing to contamination from human and zoonotic food-borne pathogens, including *Salmonella* spp., *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium botulinum*, enterohemorrhagic *E. coli* (i.e., *E. coli* O157:H7 and other Shiga toxin-producing strains of *E. coli*). Given that *Salmonella* does not grow in low a_W foods, the long-term survival of this organism in dried eggs, chocolate, hard cheese, dried infant milk, and salami has been well documented [147]. In contrast to what was commonly believed, some foodborne bacterial pathogens could survive in a dehydrated/desiccated state for long periods of time, in what was thought to be uninhabitable conditions. Some

conditions are known to enhance the microbial resistance or protect pathogens from inactivation by microbial treatments like thermal pasteurization.

Definition of Low-a_W Foods

Low-a_W foods are not always low-moisture foods. Actually, some foods with substantial amounts of moisture may have a low a_W because of water-binding solutes (e.g., sodium chloride) that bind the available water, subsequently lowering the a_W. Generally, a low-a_W food is one in which the most water present is chemically bound to solutes, making it unavailable for uptake and use by microorganisms. In food products, common water-binding solutes include sodium chloride and sucrose, whose water-binding ability is 6 times lower than sodium chloride. Water activity (a_W) is defined as the vapor pressure of a food divided by the vapor pressure of water (i.e., distilled water) at a given temperature. Pure water gives a baseline a_W reading of 1.0 under standard conditions. Therefore, all a_W measurements in foods are below 1.0. A low-a_W food has an a_W below 0.70 [148]. While low-a_W foods may also be categorized as those with a_W below 0.60, setting the boundary at <0.70 expands the category to account for various low a_W foods including certain nuts, jerkies, pepperoni, sausages, marshmallow, and nougat.

Foodborne Pathogens Associated with Low-a_W Foods

From 2007 to 2012 in the U.S., approximately 5,141 low-a_w foods were recalled due to the contamination from pathogenic bacteria [149]. In 2009 three major recalls were attributable peanut butter (3,918 products), pistachio nuts (662 products), and dry milk (286 products) which contaminated with enteric pathogens and which caused serious health and economic burdens. In 2012, peanut butter contaminated with

Salmonella Bredeney caused 42 cases of salmonellosis and a large number of recalled products [149, 150]. From 2007 to 2012, a total of 41 outbreaks involving low-a_w foods was linked 7,315 cases of reported foodborne illness resulting in 63 deaths and 536 hospitalizations [150-152].

Of the foodborne pathogens associated with enter disease from contaminated low-a_W foods, Salmonella is the most commonly implicated patyogen. Outbreaks of enteric disease linked to chocolate [153, 154], cheese [155, 156], milk powder [157], carmine [158], and paprika-powdered potato chips [159] are examples in which foods with low-a_w have been vehicles for *Salmonella* infection. *Salmonella* is widespread in nature and can survive in dry foods even for years [7, 160-164]. Its survival in foods is highly influenced by food composition, water activity, and temperature [165-169]. The pathogen shows increased resistance at decreasing water activity during heat treatment [7, 162, 170]. Additional protection against heat inactivation is given by fat in the food [171-174]. In a review [175] on the sources and risk factors of *Salmonella* in low-a_w foods, presence of the pathogen was related to cross-contamination via poor sanitation practices, poor facility and equipment design, poor maintenance, operational and manufacturing practices, as well as inadequate ingredient and pest control. [176]. *Salmonella*'s capacity to survive in dry environment may contribute to outbreaks of salmonellosis associated with low-a_w foods. *Salmonella*-related recalls and foodborne outbreaks involving low-a_W foods may be related to the unusual ability of *Salmonella* to survive in a desiccated state in low-a_W conditions, compared to other members of the *Enterobacteriaceae* family or other nonspore-forming foodborne bacteria (such as *E. coli* O157:H7 and other Shiga toxin-producing *E. coli*, *Vibrio* spp., *Listeria monocytogenes*, *Campylobacter jejuni*,

Shigella spp., *Yersinia* spp., and *Staphylococcus aureus)*. One exception is the bacterial genus *Cronobacter* (previously known as *Enterobacter sakazakii*), which caused rare but usually severe instances of foodborne illness in infants and a relatively small number of adults with severely compromised immune systems. However, in healthy adults and children, the risks of foodborne illness from *Cronobacter* spp. is low; therefore, it is not a primary threat to the general human population when compared to *Salmonella* spp. Although certain foodborne spore-forming human enteric pathogens such as *Bacillus cereus*, *Clostridium botulinum* and *Clostridium perfringens* could survive in low-a_W foods, those pathogens must multiply to relatively high cell concentrations to result in human illness via toxins or toxico-infections.

Foodborne Outbreaks Associated with Consumption of Contaminated Low-a_W Foods

A few large foodborne outbreaks have been associated with low-a_W foods in the past; however, the frequency of those types of outbreaks increased in the last two decades, presumably because of more sensitive microbial testing methods, more effective sampling plans, and improved epidemiologic trace-back investigations. A wide variety of low-a_W foods such as almonds, coconut, puffed and toasted breakfast cereals, cake mix, chocolate, cocoa, cookie dough, hazelnuts, fishmeal, halva, dried infant milk, powered infant formula, peanuts, tahini, venison jerky, black pepper, potato chips, pine nuts, powdered milk, a corn snack, salami, a rice-corn and savory snack, and aniseed tea have been implicated in foodborne illness outbreaks [147].

Pathogens implicated in those previously mentioned low-a_W foods involved with foodborne illness outbreaks include *Cronobacter* spp., *E. coli* O157:H7, *S. aureus*, *C. botulinum*, as well as the following S. *enterica* serovars with the number of outbreaks

larger than 1 associated with each serovarshown in parentheses: Agona PT15, Anatum, Derby, Durham, Ealing, Eastbourne, Enteritidis (2), Agona (4), Enteritidis PT30 and 9C, Java PT Dundee, Give, Javiana, Manchester, Montevideo, Mbandaka, Napoli, Nima, Newport, Potsdam, Oranienburg, Orion, Seftenburg Rubislaw, (2), Tennessee, St. Paul, Stanley, (2), Typhimurium, Typhi(4), Typhimurium DT104, and Wandsworth [4, 147]. Those reported outbreaks have occurred in the countries including: Austria, Australia, Belgium, China, Canada, Denmark, France, Finland, Norway, Germany, Israel, Netherlands, New Zealand, Trinidad, Sweden, UK, and USA[147]. Most outbreaks occurred in developed countries, which suggest the implementation of better surveillance systems and epidemiological methods.

Peanut butter is one example of an unusual food product to be contaminated with *Salmonella*. In 1996, an outbreak involving 15 cases of salmonellosis occurred in Australia and was traced back to *Salmonella* serovar Mbandaka-contaminated peanut butter with populations of < 4 CFU/g. In 2006-2007, a multistate foodborne outbreak of salmonellosis caused by *Salmonella* Tennessee (48 states in the U.S. with more than 700 cases of human illness) was also traced to peanut butter in manufactured over a 6-month period. ,. One year later, another salmonellosis outbreak in 46 states of US and in Canada (sickening over 700 individuals, including 9 deaths) was traced to peanut butter again. The peanut butter manufacturer in this outbreak periodically shipped contaminated peanut butter that was positive for *Salmonella* and has faced trial for criminal negligence [4].

Processing and Post-Process Contamination

Contamination of low-a_W foods with bacterial pathogen during processing and post-process is largely attributable to cross-contamination [175, 176]. Rarely, processed foods were involved in fewer US recalls (35 %) and global outbreaks (12 %). Among incriminated pathogens, *Salmonella* caused about 94 % of all low-a_W food product recalls and 53 % of total outbreaks from 2007 to 2012 [4]. As such, *Salmonella* emerges as the pathogen of greatest concern in both processed and non-processed low-a_W foods

A variety of sources and manufacturing practices has contributed to cross-contamination in the processing facilities. For example, raw materials [177] are one major source of pathogens. However, ineffective prerequisite programs including improper implementation of good manufacturing practices (GMPs) and sanitation standard operating procurers (sSOPs) at the supplier can result in pathogens being introduced into the processing facility via raw materials. Also, pathogens inevitably contaminate raw materials during growing, harvesting, and preprocessing steps in food production Apart from directly contaminating the finished food product; raw materials with pathogen could contaminate employees, equipment, and other product lines. Raw materials which are not pasteurized can contaminate finished products particularly if they serve as an ingredient subsequent to the final lethality treatment for the food product. In this regard, a pertinent example is a contaminated seasoning mix, which was added to breakfast cereals as an ingredient after a lethal heat process was applied to the cereals. The seasonings were from domestic and international suppliers [178]. Red and black pepper applied to the surface of salamis after the lethal process were the source of *Salmonella* contamination of that ready-to-eat meat product. The pepper was grown and

harvested in Asia, distributed by a US company, and finally delivered to the salami processing facility [179]. *S.* Montevideo was isolated from the crushed red and black peppers. These afore mentioned results demonstrate the high risks of cross-contamination from raw materials thus emphasizing the need for more stringent control over the microbial quality of raw materials destined for use as ingredients in processed foods.

A second major source of cross-contamination in dry foods is the environment, such as facilities and equipment, water, dust, air, insect pests and workers [147]. Employees carrying pathogens on their shoes or clothing worn outside the plant can cause cross-contamination if GMPs are not consistently followed [177, 180]. Pests such as insects and rodents that also carry pathogens may enter food-manufacturing facilities via openings of buildings, which need repair (e.g., the roofs, floors, and doors). For instance, nests of birds could introduce pathogens into the buildings through leaky roofs, or unfiltered air intake systems [177]. Contaminated food processing equipment can also harbor pathogens [177] and serve as a constant source of microbial contamination for newly processes products. Unclean vents and ventilation units with filtration systems and connected to outside environment are added sources of contamination from the external environment [177]. Multiple possible sources of *Salmonella* contamination were identified by a joint FDA and Georgia Department of Agriculture investigation of the production facility including rain and other water leakage into storage areas for roasted peanuts, and practices that allow cross-contamination between raw and roasted peanuts [181]. As revealed by the FDA investigation of the 2012 peanut butter outbreak (linked to *S.* Bredeney), raw peanuts outside the plant were in open trailers exposed to rain and on which birds were observed to land [182]. Additionally, doors of the warehouse were open

to the outside, thus facilitating the entry of pests [182]. The presence of pathogens in the processing environment and lack of water control have exacerbated the problem of product contamination from the high populations of pathogens once growth occurs.

A third major contributory factor in cross-contamination is improper facility and equipment design [147, 175, 176]. In a 2012 outbreak of *S.* Infantis infection, an inspection of the food production facility revealed that the facility was poorly designed for proper sanitation and maintaining food safety and had unclean equipment [183]. No hand washing and sanitizing facilities were found. Duct tape and cardboard were used to repair the equipment with breaks and cuts. There was a build-up of food residue and dust in equipment in areas with poor access to cleaning. Similarly, another investigation of the peanut butter processing facility involved in the 2012 outbreak of salmonellosis revealed that no hand-washing sinks were in the peanut production or packaging areas [184]. Also no documented cleaning records were available. A leaking sink in a washroom resulted in water accumulation on the floor and employees handled ready-to-eat peanuts with bare hands, and improperly handled tools and equipment [184].

Separate hygiene zones are typically established via sectioning (zoning) of a food processing facility. Zones should be set up based on an assessment of risk [147, 151, 185]. Lack of appropriate food safety controls in each zone, including the transition areas, could cause cross-contamination between the low- and high-hygiene zones [151, 185]. This includes inadequate physical separation, traffic control and airflow, dirty employee clothing, as well as cleaning and water use [147, 185]. As expected, the use of water in facilities processing of low-a_W foods is a primary risk factors for pathogen cross-contamination [147, 185]. Different water requirements are needed for different areas of a

processing facility. Therefore, most microbial control in a dry processing facility is focused on maintaining very dry conditions to limit prevent microbial growth [147, 185]. It is challenging to design a processing facility that can be properly sanitized, while maintaining separation of wet and dry areas. Bacteria on surfaces containing water are less sensitive to disinfection, mainly when present in a biofilm [186, 187]. Accordingly, after dry cleaning, an environment that seems dusty may pose a much lower risk of cross-contamination than a wet-sanitized environment without visible dust. In fact, wet cleaning facilitates the infiltration of moisture into areas that are difficult to access and to clean and may remain wet before a new round of production begins [185]. This situation ultimately results in the accumulation of high concentrations of microorganisms in hidden growth niches, which will inevitably lead to cross-contamination in the processing facility.

For low-a_W foods that receive a lethal process, inadequate process control is a major factor related to pathogen contamination [180, 188]. Generic lethality processing standards for foods with high moisture should not be used for low-a_W foods, since they may lead to inadequate pathogen inactivation [180]. For example, in the investigation of *Salmonella* outbreak in Georgia processed peanuts in 2008–2009, there was debate regarding whether the peanut roaster reached a temperature high enough to eliminate *Salmonella* routinely [181].

Current Challenges in the Control of Foodborne Pathogens in Low-Water Activity Foods

The strict control of foodborne pathogens in low-a_W foods is highly challenging. Raw materials are in several instances produced under conditions in which the entry of pathogens into the food is unavoidable. Dry foods could be contaminated with pathogens

at harvest and storage and in preprocessing environments. Control of dust, pests, and water is crucial for pathogen control in these foods. Facility and equipment design might be inappropriate for control of these factors and conventional wet cleaning practices may enhance pathogen survival and growth due to the lack of water control. Several low-a_W food products are ready to eat, not cooked before consumption, and have a long shelf life; therefore, high standards of hygiene are needed for the production process. Parameters for inactivation of pathogens in processing dry foods are usually not well defined. Consequently, several dry food products have no history of undergoing validated pathogen-destruction processes. The fact that pathogens survive during storage of foods for extended periods and these foods typically have a long shelf life substantially increases their risks to public health [4].

Processing Practices to Reduce Pathogen Contamination in Low-Water Activity Foods

The Grocery Manufacturers Association (GMA) has made guidelines to limit *Salmonella* in processing facilities and promote the microbial safety of low-a_W foods [189]. Meanwhile, an industry handbook about safe processing of nuts has been developed [190] as well as equipment [191] and facility [192] design checklists to self-evaluate compliance along with GMA sanitary design principles. Furthermore, in view of the foodborne illnesses burdens in the US, the FDA has launched the Food Safety Modernization Act (FSMA) rule, which has been signed into law in 2011. By using a risk-based approach, this law transfers the focus of regulators from responding to an event to preventing one from taking place [193]. Guidance for industry and rules related to the FDA FSMA contains preventive controls for human and animal food, produce food safety standards, a foreign supplier verification program, and third-party certification

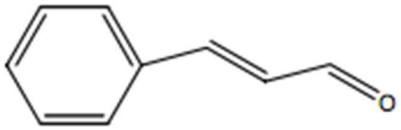
[193]. Guidance documents advise food processors to evaluate the risk that ingredients might carry pathogens. In this regard, many questions on the nature of the product, the type of treatment, the use of the product, whether the process to eliminate pathogens was validated, and the relevant cooking instructions must be made for consumers [147]. Good agricultural, manufacturing, and hygienic practices need to be utilized at each step in the processing/manufacturing chain. In the processing facility, separate hygiene areas must be established according to the requirements for moisture control and exposure of product to the environment, and a monitoring program for the environment must be developed for the pathogens with highest risk. Hygienic principles of equipment design and installation should meet the need for water control and dry/wet cleaning.

Cinnamaldehyde

Cinnamaldehyde, also known as (2E)-3-phenlprop-2-enal, was isolated for the first time in 1834 by Dumas and Peligot from cinnamon. Cinnamaldehyde is a pale yellow, viscous liquid that is present naturally in cinnamon trees and species of the genus *Cinnamomum*. It is responsible for cinnamon's unique flavor and odor. Cinnamaldehyde makes up 1-8%, 55-75%, and 70-95% of essential oil acquired from cinnamon leaf, cinnamon bark, and cassia, respectively[194]. Under the Code of Federal Regulations Title 21, part 182, cinnamaldehyde is a flavoring species normally recognized as safe. Apart from being used as a flavoring agent, cinnamaldehyde is widely used in fragrances present in cosmetics, shampoos, fine fragrances, as well as cleaners and detergents [195]. Therefore, cinnamaldehyde is a natural product with multifunctional applications and more research is needed to expand the scope of its antimicrobial properties.

Chemical Structure

Cinnamaldehyde (molecular formula is C_9H_8O) has a molecular weight of 132.2 g/mol. Upon extraction from cinnamon, it is commonly referred as trans-cinnamaldehyde (Figure 2). Trans-cinnamaldehyde consists of a phenyl group adjunct to an aldehyde moiety and makes up ca. 65% of cinnamon [196].



Chemical structure of cinnamaldehyde. (Adapted from [195])

Antimicrobial properties of cinnamaldehyde and mechanism

Aldehyde units are chemically reactive and can cross-link with DNA proteins covalently via amino groups and alter their normal functions [197]. Cinnamaldehyde exhibited high antimicrobial activity towards microorganisms [198]. In 1887, Chamberland demonstrated that cinnamon oil was lethal against anthrax spores [199]. Cinnamon displayed inhibitory effects against fungi and bacteria compared to the extracts of herbs and spices at a variety of pHs [200]. Despite the inconclusive mode of action of cinnamaldehyde, it is widely believed that three reactions could potentially occur, 1) cinnamaldehyde inhibits many enzymes involved in cytokinesis at low concentrations, 2) at larger, yet sub-lethal concentrations, cinnamaldehyde behaves as an ATPase inhibitor, 3) at lethal concentrations, cinnamaldehyde disrupts the cell membrane [201]. As for *Bacillus cereus*, it inhibited cytokinesis as a mode of action owing to cells not separating although septa existed after division [201]. Also cinnamaldehyde could bind to the FtsZ protein, and inhibit its GTP dependent polymerization in turn, leading to prevention of cell division [202, 203]. Cinnamaldehyde could inhibit the growth of *E. coli* O157:H7

and *Salmonella typhimurium* at similar concentrations to that of carvacrol and thymol; Nevertheless, cinnamaldehyde failed to disrupt the outer membrane or exhaust the intracellular ATP pool [204]. Cinnamaldehyde dramatically decreased the viability of *Helicobacter pylori* at a concentration of 2 μg/mL [205]. Furthermore, cinnamaldehyde could inhibit amino acid decarboxylases in *Enterobacter aerogenes* as well. The underlying mechanism is possibly due to the carbonyl moieties binding to proteins, thereby preventing the further action of amino acid decarboxylases [206].

At sub lethal concentrations, cinnamaldehyde can enter the periplasm and inhibit the activity of transmembrane ATPase [201]. As demonstrated by a previous work [204], trans-cinnamaldehyde was utilized to test its inhibitory effect against *E. coli* O157:H7 and *Salmonella typhimurium*. Additionally, its toxicity to *Photobacterium leiognathi* was investigated as well. It was found that trans-cinnamaldehyde could inhibit *E. coli* and *Salmonella* at 3 mM. Notably, trans-cinnamaldehyde was able to inhibit enterobacterial growth and bioluminescence of *P. leiognathi* without causing the outer membrane permeable. Therefore, the trans-cinnamaldehyde can acquire access to the periplasm and inner parts of the bacterial cell. Gill and Holley [207, 208] proved the capacity of cinnamaldehyde to access the periplasm by revealing that the increasing concentrations of this essential oil component (13.6-1362 μg/ml) can decrease ATPase activity of isolated cell membranes. However, cell death, was not specifically ascribed to ATPase inhibition since the concentration of cinnamaldehyde (681-1362 μg/mL) necessary to inhibit ATPase lead to membrane disruption of *E. coli* cells as well [207].

Though quite a few studies have indicated that cinnamaldehyde interacts with the cell membrane, it is still unclear how membrane disruption occurs. Di Pasqua et al. [209]

found that the general mechanism of cinnamaldehyde action was not membrane disruption. Upon the introduction to to *E. coli*, *S. enterica*, *P. fluorescens*, and *B. thermosphacta*, cinnamaldehyde led to remarkable increases in saturated fatty acids, thus changing the membrane lipid shape [209, 210]. The end result was a more rigid membrane and altered cell structure. As for *S. aureus*, the cell envelope disintegrated. In the test against fungi, the primary mechanism of cinnamaldehyde's action is thought to be cell division inhibition. Bang et al. [211] confirmed that cinnamaldehyde, acting as a non-competitive inhibitor of β-(1,3)-glucan synthase and a mixed inhibitor of chitin synthase isozymes in *Saccharomyces cerevisiae*, inhibited cell wall synthesizing enzymes.

As demonstrated with geranial, cinnamaldehyde acted synergistically against bacteria upon the combination with certain plant extracts. Moleyar and Narasimham [212] revealed that a mixture of cinnamaldehyde and eugenol at concentrations of 250 and 500 μg/mL, completely inhibited growth of *Bacillus* sp., *Staphylococcus* sp., *Micrococcus* sp. and *Enterobacter* sp. for more than one month. When the antimicrobials were tested separately, no growth inhibition was observed. Likewise, in another separate study, cinnamaldehyde mixed with thymol or carvacrol efficiently inhibited the growth of *Salmonella* Typhimurium in Mueller Hinton broth [213].

The stability of essential oils and their components during food processing requires further investigation. When heated alone, cinnamaldehyde decomposed to benzaldehyde at 60 °C. However, when combined with eugenol or cinnamon leaf oil, cinnamaldehyde was thermally stable up to 200°C for 5 hours [214]. Cinnamaldehyde hold vast potential as an antimicrobial material in spite of several studies debating its capacity to disrupt bacterial cell membranes. Further studies are highly desired to

uncover the full potential of cinnamaldehyde as well as the underlying mechanism of action.

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CHAPTER 3 ULTRAVIOLET RADIATION RESISTANCE AND INJURY OF LONG-TERM-SURVIVAL PHASE *SALMONELLA* TYPHIMURIUM ATCC 14028 IN 0.85% SALINE AND APPLE JUICE

A paper to be submitted to Foodborne Pathogens and Disease

Fei Wang[4], Aubrey Mendonça[1,4]*, Byron Brehm-Stecher[1,4], James Dickson[2,4], Alan DiSpirito[3,4], Angela Shaw[1], and Emalie Thomas-Popo[1,4]

[1]Department of Food Science and Human Nutrition, [2]Department of Animal Science, [3]Department of Biochemistry, Biophysics, and Molecular Biology, and [4]Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, Iowa 50011

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-2950. Fax: (515) 294-8181. Email: amendon@iastate.edu.

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Abstract

Non endospore-forming pathogenic bacteria in the long-term-survival (LTS) phase can remain viable for months or years and may develop resistance to antimicrobial interventions. In the present study, we investigated the resistance of LTS phase *Salmonella enterica* serovar Typhimurium (ATCC 14028) to ultraviolet (UV) radiation in 0.85% (wt/vol) saline and apple juice, as well as the extent of sub-lethal injury in LTS phase survivors. The LTS phase *S.* Typhimurium cells were cultured in tryptic soy broth with 0.6% yeast extract (TSBYE) at 35°C for 14 days. Exponential- and stationary-phase cells, cultured in TSBYE (35°C) for 4 h and 24 h respectively, served as control samples. Cells (10^7 CFU/mL) from each physiological state were exposed to UV light in saline (80 μW/cm^2) and apple juice (1500 μW/cm^2). The *S.* Typhimurium survivors were enumerated by plating diluted (10-fold) samples on tryptic soy agar with 0.6% yeast extract or xylose lysine tergitol agar and counting bacterial colonies after incubation (35°C, 24 h). LTS phase cells consistently exhibited the highest UV resistance ($P < 0.05$). In saline, D-values of exponential, stationary, and LTS *S.* Typhimurium were 0.35, 0.38 and 0.49 min, respectively. In apple juice (pH 3.63) D-values were 2.52, 3.19, and 3.57 min, respectively; in pH-adjusted apple juice (pH 5.65) D-values were 3.24, 3.50 and 4.18 min, respectively ($P < 0.05$). UV radiation (80 μW/cm^2) of *S.* Typhimurium in saline for 2.5 min reduced the number of exponential- and stationary-phase cells by ~7.19 and 6.30 log CFU/mL, respectively. In contrast, LTS cells were only reduced by 5.08 log. Among the three physiological states, LTS phase cells had the least sub-lethal injury in the surviving population ($P < 0.05$). These results indicate that the LTS state cross-protects *S.* Typhimurium against UV radiation and should be considered in determination of the UV radiation D-value for this pathogen.

Introduction

Salmonellae are Gram-negative rod-shaped facultative anaerobic bacteria that are a serious public health concern worldwide. Among foodborne pathogenic bacteria *Salmonella enterica* are a major cause of the largest number of food-related deaths with the highest cost burden in the United States (Batz, Hoffmann, & Morris Jr, 2012). Recent estimates indicate that in 2013 the total annual cost of foodborne illness from *Salmonella* was about $3.6 billion (ERS). Internationally, gastroenteritis is characteristic of foodborne salmonellosis accounting for 93.8 million cases and resulting in about 155,000 deaths per year (Majowicz, Musto, Scallan, Angulo, Kirk, O'brien, et al., 2010). While humans with can harbor typhoid salmonellae (*Salmonella* Typhi and *Salmonella* Paratyphi) during acute or chronic infections, birds, reptiles, amphibians, and feral, farm and domestic animals are major reservoirs of non-typhoid *Salmonella* (NTS). *Salmonella* shed in animal feces can survive for long periods in the natural environment and the prevalence of *Salmonella* in farm environments has been reported to range from 10 to 26% (Rodriguez, Pangloli, Richards, Mount, & DRAUGHON, 2006). The major transmission route for NTS infection to humans is fecal-oral via consumption of contaminated food or water. Due to numerous animal reservoirs of NTS and its widespread distribution in the environment *Salmonella* can remain a persistent hazard in a wide variety of raw foods, including red meats, poultry, milk, eggs, fish and shellfish, cereals, vegetables, fruits and fruit juices (Noël, Hofhuis, De Jonge, Heuvelink, De Jong, Heck, et al., 2010; Pui, Wong, Chai, Nillian, Ghazali, Cheah, et al., 2011).

Ultraviolet (UV) irradiation is an attractive alternative process for juices which inevitably undergo nutritional and sensory changes during heat pasteurization. When compared with heat pasteurization (high temperature short time) for liquid foods, UV

treatment had minimal effect on the quality characteristics of juice (Noci, Riener, Walkling-Ribeiro, Cronin, Morgan, & Lyng, 2008). The U.S. Food and Drug Administration (FDA) approved UV irradiation as a cost-effective alternative to thermal pasteurization of fresh juice (Donahue, Canitez, & Bushway, 2004; Food & Drug Administration, 2012). During the UV irradiation process the treated food product is exposed to a germicidal light with a wavelength of 220–300 nm to inactivate microorganisms. Exposure to UV radiation dimerism adjacent pyrimidine nucleotide bases on the same DNA or RNA strand thus rendering microorganisms incapable of replicating their DNA which in turn leads to microbial inactivation and inability to cause infection (Sizer & Balasubramaniam, 1999). The extent of inactivation of foodborne pathogens is directly dependent on the UV radiation dose applied to the food products.

The effect of growth phase on bacterial resistance to UV radiation as well as ionizing radiation (electron-beam) has been reported. For example, *Escherichia coli* cells in the stationary phase exhibited an increased resistance to UV radiation compared to exponential phase cells (Abedi-Moghaddam, Bulic, Herderson, & Lam, 2004). Stationary phase *Listeria monocytogenes* cells were more resistant than exponential phase cells to electron beam irradiation in 0.85% saline (NaCl) and in ground pork (Mendonca, Romero, Lihono, Nannapaneni, & Johnson, 2004). The higher resistance of stationary phase bacteria to external stresses has been attributed to bacterial expression of specific stress proteins (Boor, 2006; Price, Fawcett, Cérémonie, Su, Murphy, & Youngman, 2001). In this regard stationary phase bacterial cells are routinely used as target organisms in challenge studies to validate the antimicrobial efficacy of food processes.

The scientific literature is replete with information on four phases of the bacterial life cycle. In a review of the stationary phase of bacteria, Kolter et al. (Kolter, Siegele, & Tormo, 1993) describes four phases, namely, the lag phase, the exponential phase, the stationary phase, and the death phase. However, some researchers have discussed a fifth phase in which bacteria display long-term survival (LTS). This fifth phase occurs after the death phase and is called the "senescent phase" in *Serratia* and *Sarcina* (Steinhaus & Birkeland, 1939) and "long-term stationary phase" in *E. coli* (Finkel, 2006). The LTS phase was described in *L. monocytogenes* whereby the viable cell density of the pathogen remained at 10^8 CFU/mL for over 30 days in tryptic soy broth with added yeast extract at 35 ºC (Wen, Anantheswaran, & Knabel, 2009). Those same researchers reported that LTS *L. monocytogenes* were predominantly coccoid in shape and exhibited significantly greater resistance to heat and high pressure compared to both stationary- and exponential phase cells.

While there is an emerging body of knowledge on the enhanced resistance of LTS *L. monocytogenes* to food processes such as thermal processing and high hydrostatic pressure (Wen, Anantheswaran, & Knabel, 2009), there are no published reports describing the LTS phase in *Salmonella* and the effect of that phase on the pathogen's resistance to a non-thermal process such as UV irradiation. Accordingly, the purpose of the present study was to evaluate the resistance of LTS *Salmonella* Typhimurium ATCC 14028 to UV radiation in 0.85% saline and in apple juice. An additional objective was to compare the extent of sub-lethal injury in exponential-, stationary- and LTS phase survivors of *S.* Typhimurium following UV radiation in 0.85% saline and in apple juice.

Materials and Methods

Bacterial strain and culture conditions

Salmonella enterica serovar Typhimurium ATCC 14028 used in this study was obtained from the culture collection of the Microbial Food Safety Laboratory of Iowa State University. The culture was maintained as frozen (-80°C) stock in brain heart infusion (BHI) broth (Difco; Becton Dickinson, Sparks, MD) containing 10% (v/v) glycerol. The frozen stock culture, thawed under cold running water, was activated in tryptic soy broth (TSB; Difco) supplemented with 0.6% yeast extract (TSBYE) at 35 °C. Prior to each experiment at least two consecutive 24-h transfers of the activated stock culture were performed in TSBYE (35 °C) to prepare a working culture.

Exponential, stationary, and long-term survival cells

A portion (1.0 mL) of *S.* Typhimurium ATCC 14028 working culture was transferred to TSBYE (100 mL) in a screw-capped 250 mL Erlenmeyer flask. The inoculated medium was incubated at 35°C with shaking (150 rpm) in a gyrorotary shaker incubator (New Brunswick Scientific Co. Inc., Edison, NJ) for 2.5 h and 18 h to obtain exponential- and stationary phase cells, respectively. *S.* Typhimurium was cultured in TSBYE (100 mL) at 35°C for 15 to 30 days to obtain long-term survival (LTS) cells.

Determination of cell viability

Serial dilutions (10-fold) of *S.* Typhimurium cultures in each of the three physiological states (exponential, stationary and LTS) were prepared in buffered peptone water (BPW, Difco), and 0.1-mL aliquots of appropriate dilutions were surface plated on both tryptic soy agar (TSA; Difco) supplemented with 0.6% yeast extract (TSAYE; Difco) and xylose lysine tergitol agar (XLT4; Difco). All inoculated agar plates were

incubated aerobically at 35°C and the bacterial colonies were counted at 24 h to determine the concentration of viable cells in each physiological state.

Preparation and inoculation of saline and apple juice

Pasteurized, clarified apple juice (pH 3.63) was purchased from a locally in Ames, Iowa and the pH of a portion of that juice was adjusted to pH 5.65 with 1M aqueous NaOH solution. The juice was sterilely filtered using a bottle-top vacuum filtration system with 0.22 µm pore size filter (Corning, Amsterdam, Netherlands). Samples of filter-sterilized apple juice were stored at 4 °C before inoculation. Exponential, stationary and LTS cells in TSBYE (35°C) were harvested by centrifugation (10,000 x g, 10 min, 22 ºC) using a Sorvall Super T21 ultra centrifuge (Sorvall Product, L.P., Newtown, CT). The pelleted cells were washed (by vortexing) in 0.85% (w/v) NaCl (saline), harvested by centrifugation (10,000 x g, 10 min, 22 ºC), then suspended in appropriate volumes of fresh saline or sterilized apple juice (pH 3.63 or 5.65) to obtain a final cell suspension of ~ 10^7 CFU/mL.

UV irradiation treatment

The two UV radiation intensities (µW cm^{-2}) used in this study were achieved by adjusting the distance between the UV lamp and samples, and measuring the intensity using an ultraviolet meter (Model DM-254XA, Spectronics Corporation, Westbury, New York, USA). The UV lamp used was a Spectroline Model XX- 15F (Spectronics Corporation, Westbury, New York, USA) emitting short wave UV light (254 nm), and working at 120 volts, 60 Hz and 0.7 amps. Aliquots (5-mL) of *S.* Typhimurium cell suspensions in either saline or apple juice were dispensed into sterile 60×15 mm plastic petri dishes (Falcon, Tewksbury, MA). All samples of cell suspensions (each 2.0 mm deep) in petri dishes were positioned on a stirrer (Thermolyne Cimarec 2, Dubuque,

Iowa) and stirred at rate of 5 revolutions per minute (rpm). Cell suspensions in saline were exposed to a UV radiation intensity of 80 μW cm^{-2} for 0 (control), 30, 60, 90, 120, 150, and 180 s whereas, cell suspensions in apple juice were exposed to1500 μW cm^{-2} for 0 (control), 2, 4, 6, 8, 10, and 12 min. Stable UV radiation intensity was achieved by warming up the UV lamp for 10 min prior to each experiment.

Microbiological analysis

Serial dilutions (10-fold) of control and UV-treated samples of *S.* Typhimurium in saline and apple juice were prepared in BPW and aliquots (1.0- or 0.1-ml) of appropriate dilutions were surface-plated, in duplicate, on TSAYE and XLT4 plates. In instances when populations of *S.* Typhimurium survivors were less than 10 CFU/mL, 1-mL samples of non-diluted saline or apple juice were plated directly on agar media. All inoculated agar plates were incubated at 35 °C and bacterial colonies were counted at 24 h.

Calculation of D-values

To calculate D-values, survivor curves were prepared by plotting numbers of *S.* Typhimurium survivors (log_{10} CFU/mL) versus time of exposure (min) to UV radiation using Microsoft Excel 2010 Software (Microsoft Inc., Redmond, WA). The line of best fit for the data was obtained by linear regression analysis (Ostle et al., 1975). The D-values (exposure times at a specified UV radiation intensity that produce a 90% reduction in the initial population of viable count of *S.* Typhimurium) were determined by calculating the negative reciprocal of the slopes of the regression curves.

Determination of sub-lethal injury

For each replicate experiment, survivor curves based on recovery of bacterial colonies on TSA and XLT4 were prepared. Viability reduction in S. Typhimurium was

expressed as the logarithm of the reduction factor (RF). The RF is the ratio of the colony counts (CFU/mL) of untreated control to the CFU/mL of the treated sample (Wuytack, Phuong, Aertsen, Reyns, Marquenie, De Ketelaere, et al., 2003). The log of the RF from CFU on XLT4 plates was plotted on the y-axis against the log of the RF from CFU on TSAYE plates on the x-axis, and linear regression lines were fitted through the data points. The extent to which each treatment caused sub-lethal injury was compared from the slopes of the regression lines for each treatment (Wuytack, et al., 2003).

Statistical analysis

All experiments were replicated three times and results are reported as averages. The D-value data and linear regression model were made using SAS software (SAS version 8.2, SAS Institute, Cary, N.C.). Tests were carried out at a 5% significance level.

Results and discussion

Cell viability at growth phases

Figure 1 shows growth of *S.* Typhimurium ATCC 14028 in TSBYE (35°C) at different times to produce cells in exponential phase (A), stationary phase (B), death phase (C) and long-term-survival phase (D). The exponential phase lasted for ~13 h until the population reached ~9.82 log CFU/mL (stationary phase; B). The duration of the stationary phase was about 24 h before the cells entered into death phase (C) and the viable population decreased to ~ 7.90 and 6.50 log CFU/mL at about 2 and 30 days, respectively. After 30 days, numbers of viable cells remained relatively stable in TSBYE for more than 36 months without being supplied with added nutrients. When *S.* Typhimurium cells entered the LTS phase there was no significant different between viable counts (CFU/mL) obtained at set times from 30 to 1200 days ($P > 0.05$). This pattern of growth phases, including the long-term-survival (LTS) phase, is consistent

with that reported by Finkel (Finkel, 2006) for *Escherichia coli* growing in Luria Bertani (LB) medium at 37 °C. When *S.* Typhimurium cells entered the LTS phase there was no significant different between viable counts (CFU/mL) obtained at set times from 30 to 1200 days ($P > 0.05$). Even though a few minor aspects such as length of the lag- and exponential phases and highest cell concentration attained, may vary due to several factors such bacterial species or the growth conditions, the general profile of the five-phase life cycle for bacteria is similar. In the LTS phase viability of bacteria can remain constant for months or even years (Finkel, 2006).

UV resistance in saline and apple juice

Figure 2 shows survival curves of exponential-, stationary- and LTS phase cells of *Salmonella* Typhimurium ATCC 14028 following UV irradiation (80 $\mu W/cm^2$) for 3 minutes in 0.85% (wt/vol) saline at 23 ± 1°C. Irrespective of growth phase, cell viability declined with increasing exposure to UV radiation. Compared to exponential- and stationary phase cells, a higher concentration of LTS cells of the pathogen remained viable after 2.5 and 3.0 minutes. After 2.5 minutes of no exponential cells could be detected (detection limit 1 CFU/ml) whereas numbers of stationary phase and LTS survivors were 1.20 and 2.60 log CFU/mL, respectively. After 3.0 minutes neither exponential- or stationary phase cells could be detected, representing a 7.0 log CFU reduction in initial numbers of the pathogen. In contrast about 2.04 log CFU of LTS survivors remained reflecting a 4.96 log CFU reduction. Radiation (UV) D-values, derived from slopes of the regression lines for survivor cells from the three growth phases indicate that the D-value for LTS cells was significantly higher ($P < 0.05$) than the D-value for exponential- or stationary phase cells in 0.85% saline (Table 1).

This pattern of higher resistance of LTS cells to UV radiation (1500 μW/cm^2) was also observed in apple juice (Table 1). In both apple juice at pH 3.63 and pH-adjusted apple juice (pH 5.65), the D-values for LTS cells were significantly higher ($P < 0.05$) than those of exponential- and stationary phase cells (Table 1). For both 0.85% saline and apple juice the order of UV radiation resistance of *S.* Typhimurium cells based on D-values was as follows: exponential phase cells < stationary phase cells < LTS phase cells ($P<0.05$). After 4 through 12 minutes of UV irradiation in apple juice, irrespective of juice pH, log CFU reduction in viable counts was significantly lower ($P< 0.05$) for LTS cells compared to exponential- or stationary phase cells (Figure 3 A and B).

These results indicate that *S.* Typhimurium in the LTS state has a greater resistance to UV radiation compared to exponential- or stationary phase cells of that pathogen. While to date there are no published reports on the resistance of LTS phase *S. enterica* to UV radiation, our results are consistent with those of Wen et al (Wen, Anantheswaran, & Knabel, 2009) who demonstrated significantly ($P <0.05$) enhanced resistance of LTS *L. monocytogenes* to high pressure and thermal treatment compared to the pathogen in other phases (late exponential-, stationary-, early death-, or late death phase) of the life cycle (Wen, Anantheswaran, & Knabel, 2009). Also, bacteria that cease growing and remain viable without nutrients for extended periods of time seem to develop enhanced resistance to antimicrobial interventions. For example, washed cells of *E. coli* O157:H7 and *L. monocytogenes* Scott A that survived for 10 days in 0.85% saline exhibited significantly ($P <0.05$) enhanced resistance to electron beam radiation compared to washed exponential – and stationary phase cells harvested from TSBYE

(Hong, Mendonça, Daraba, & Shaw, 2014; Mendonca, Romero, Lihono, Nannapaneni, & Johnson, 2004).

In the natural environment, conditions that allow constant growth of bacteria as observed in nutrient-rich laboratory broth media are rarely found. In fact, due to limited nutrients and harsh conditions in natural environments, bacteria may be forced to enter a prolonged stationary phase in which they exhibit a low metabolic rate or dormancy (Kolter, Siegele, & Tormo, 1993). For example, cell dormancy induced by nutrient limiting conditions has been reported in marine bacteria (Novitsky and Morita). While some species of Gram-positive bacteria produce dormant spores, many Gram-negative bacteria produce resistant vegetative cells that are seemingly dormant. Therefore, it is likely that LTS cells enter a dormant state that contribute to their reduced sensitivity to antimicrobial interventions. This explanation seems plausible because "persister" cells of clinically important bacteria are described as dormant, non-dividing cells with reduced metabolism and those cells have a very high antibiotic tolerance (Lewis, 2010; Shah, Zhang, Khodursky, Kaldalu, Kurg, & Lewis, 2006). In this regard, further research is needed to determine whether or not LTS cells of *S.* Typhimurium are actually in a dormant state.

UV radiation induced sub-lethal injury

Table 2 shows, as an index of sub-lethal injury, the linear regression slopes from plots of viability reduction for exponential, stationary and long-term-survival cells of *S.* Typhimurium on selective versus non-selective media following UV irradiation (1500 $\mu W/cm^2$) in apple juice. The logarithm of the viability reduction, as calculated for XLT4 selective agar, was plotted on the y axis against the log of the viability reduction for the non-selective TSAYE agar on the x axis and linear regression lines were fitted through

the data points (Wuytack, et al., 2003). The degree to which each treatment causes sub-lethal injury was compared from the slopes of the lines that are listed in Table 2. When the slope is equal to 1, there is no sub-lethal injury, since the same viability reduction is observed on both selective and non-selective agars. In contrast, a slope that is >1 indicates sub-lethal injury, since a higher viability reduction is observed on the selective plates than on the non-selective plates. The more the slope deviates from 1, the higher the extent of sub-lethal injury.

The *S.* Typhimurium cells from all three growth phases exhibited less sub-lethal injury in pH-adjusted apple juice (pH 5.65) compared to apple juice with pH of 3.63; however, this difference in sub-lethal injury was only significant ($P < 0.05$) for exponential- and stationary phase cells but not for LTS cells (Table 2). In the pH-adjusted apple juice (pH 5.65) the difference in extent of sub-lethal injury in stationary phase cells and LTS cells was not significant ($P > 0.05$). The highest and lowest levels of sub-lethal injury were observed in exponential phase cells and LTS cells, respectively irrespective of the juice pH ($P < 0.05$).

In the present study sub-lethally injured cells were those that, at the time of analysis, formed colonies on non-selective agar (TSAYE) but failed to do so on selective agar (XLT). Such cells most likely endured UV radiation-induced damage which they were unable to repair on selective agar. The primary cellular target of UV radiation is the DNA in which adjacent pyrimidines on the same DNA strand form dimers that stop DNA replication and consequently cell division (Lindahl, 1993). Similar to our findings, Abedi-Moghaddam et al (Abedi-Moghaddam, Bulic, Herderson, & Lam, 2004) reported that stationary *E. coli* cells were more resistant than exponential cells to UV radiation.

Those authors suggested the involvement of an up-regulation of DNA repair mechanisms during stationary phase or a lower rate of DNA replication, which permits more time for DNA repair in stationary phase cells. Based on the results of the present study, the LTS cells either have a way to protect their DNA from UV-radiation-induced damage or have more efficient repair systems to eliminate the damage. Alternatively, LTS cells most likely have no DNA replication thus allowing more time for DNA repair.

Conclusions

Based on the results of this study the LTS state substantially increases the resistance of *S.* Typhimurium to UV radiation and permits less sub-lethal injury in those cells compared to exponential or stationary phase cells. Stationary phase bacterial cells are typically used in validation food processes for inactivation of foodborne pathogens. However, the findings of the present study indicate that stationary phase *S.* Typhimurium ATCC 14028 cells are significantly less resistant to UV radiation than cells in the LTS phase. Consequently, food processing or preservation methods based on inactivation studies involving stationary phase cells may be inadequate to reduce the food safety risk from *Salmonella* due to an overestimation of the antibacterial effect of the methods. Salmonellae may survive in a LTS state in food processing environments particularly in hard-to-clean growth niches (Tompkin, 2002) or in biofilms (Rodrigues, Teixeira, Oliveira, & Azeredo, 2011) and become a constant source of contamination to food products. Additionally, LTS-induced protection of pathogens against food UV irradiation treatment should be considered when determining D-values to ensure the microbial safety of foods treated with UV radiation.

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Figures and Tables

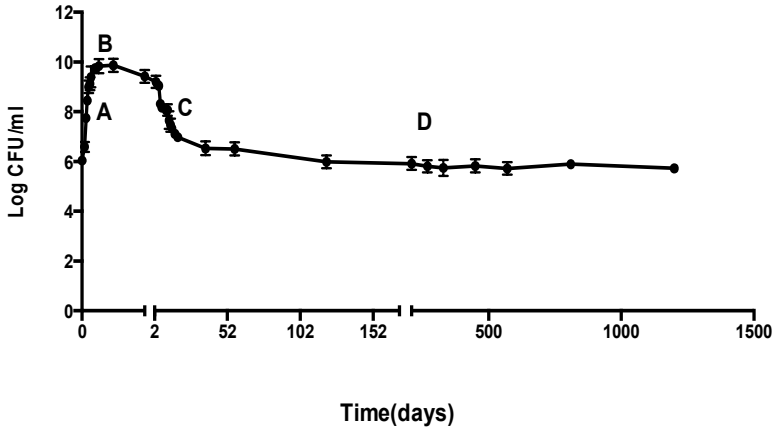

Figure 1. Growth of *S.* Typhimurium (ATCC 14028) in TSBYE at 35°C for different times to yield exponential-phase (A), stationary-phase (B), death-phase (C), or long-term-survival phase (D) cells. The data points and error bars represent averages and standard deviations, respectively, based on three replications of the experiment.

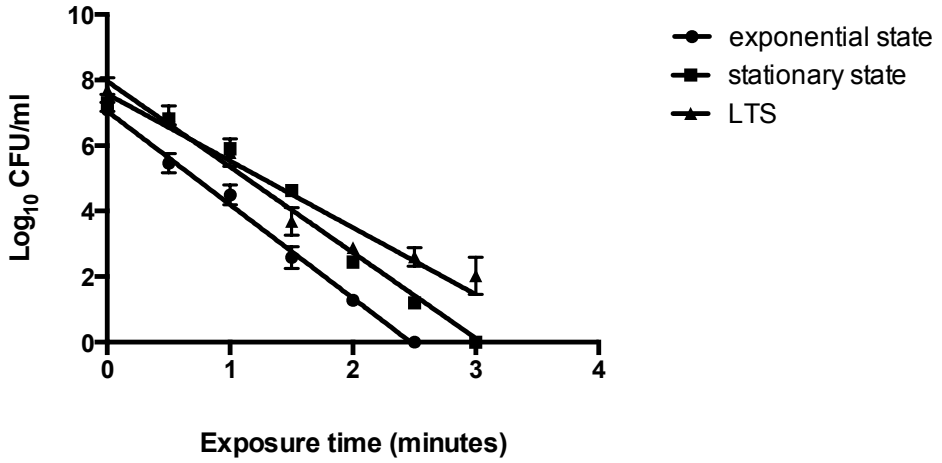

Figure 2. Survival of exponential-, stationary- and long-term-survival phase cells of *Salmonella* Typhimurium ATCC 14028 following UV irradiation in 0.85% (wt/vol) saline at 23 ± 1°C. The data points and error bars represent averages and standard deviations, respectively, derived from three replications of the experiment.

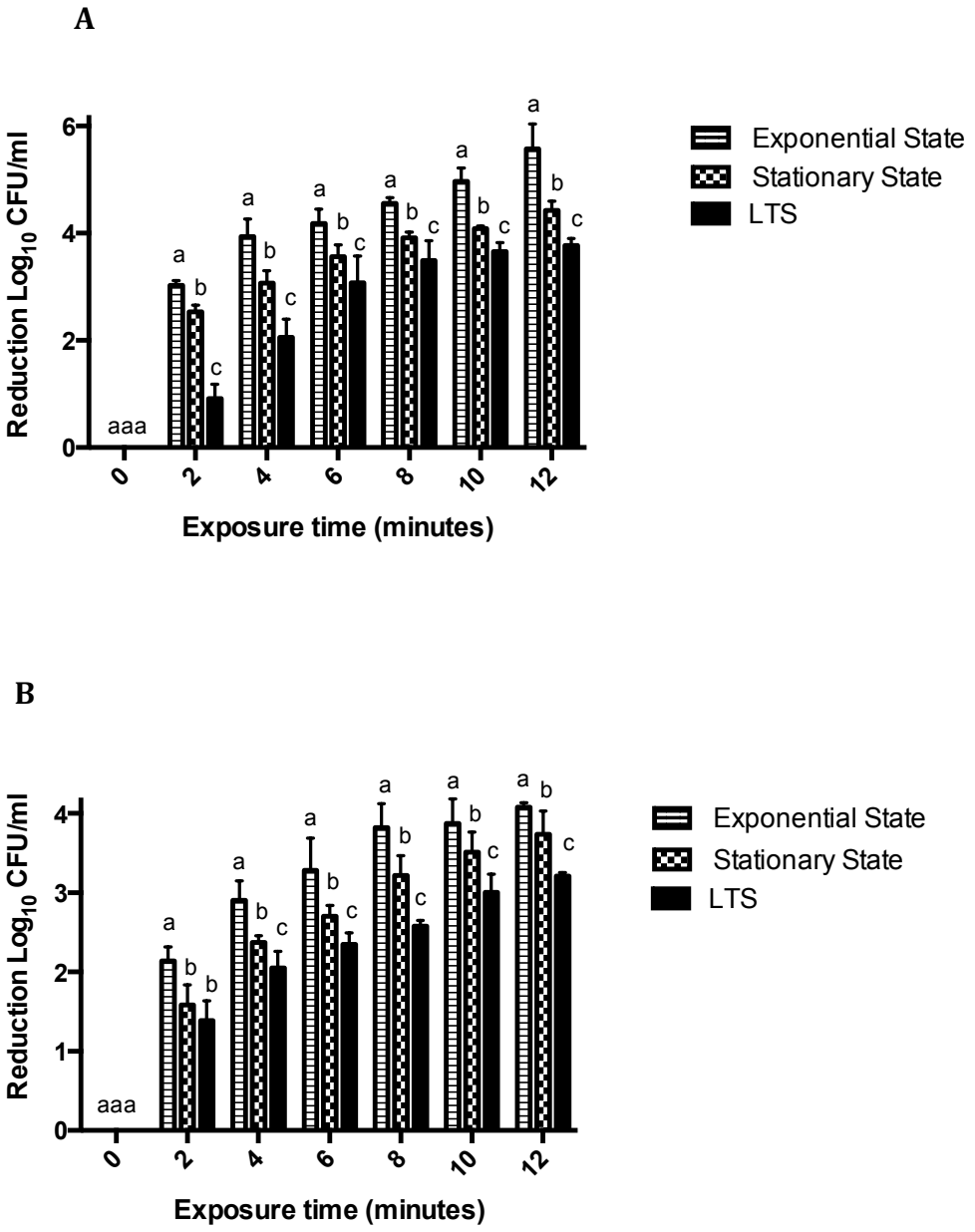

Figure 3. Reduction of exponential, stationary and long-term-survival cells of *S.* Typhimurium following UV irradiation in apple juice (pH 3.63, **A**) and pH-adjusted apple juice (pH 5.65, **B**). Reductions in populations are average values from three replications of the experiment. Error bars represent standard deviations. For each exposure time point, bars that do not share a common letter are significantly different ($p < 0.05$).

Table 1. Radiation (UV) D-Values (minute) for exponential, stationary and long-term-survival cells of *Salmonella* Typhimurium in 0.85% saline, apple juice (pH 3.63) and pH-adjusted apple juice (pH 5.65)

Growth phase	0.85% Saline 80 µW/cm^2	Apple Juice (pH 3.63) 1500 µW/cm^2	Apple Juice (pH 5.65) 1500 µW/cm^2
Exponential	0.35±0.00Ax	2.52±0.12Ay	3.24±0.06Az
Stationary	0.38±0.00Bx	3.19±0.05By	3.50±0.07Bz
LTS	0.49±0.01Cx	3.57±0.06Cy	4.18±0.06Cz

Survivors were recovered on tryptic soy agar supplemented with 0.6% yeast extract.

Values are averages ± standard deviations from three replications of the experiment.

All averages are within each column (A, B, C) or within each row (x, y, z) are significantly different ($P < 0.05$).

Table 2. Linear regression slopes from plots of viability reduction (reduction factor) for exponential, stationary and long-term-survival cells of *Salmonella* Typhimurium on selective versus non-selective media following UV irradiation (1500 $\mu W/cm^2$) in apple juice.

Growth Phase	**Apple Juice (pH 3.63)**	**Apple Juice (pH 5.65)**
Exponential	$1.16 \pm 0.11^{Aa*}$	1.04 ± 0.07^{Cb}
Stationary	1.10 ± 0.01^{Ba}	1.00 ± 0.09^{Db}
Long-term-survival	1.05 ± 0.02^{Ca}	0.99 ± 0.04^{Da}

Average values with the same uppercase letter in the same column or with the same lowercase letter in the same row are not significantly different ($P > 0.05$)

*Values are averages ± standard deviations from three replications of the experiment.

CHAPER 4 DESICCATION RESISTANCE OF LONG-TERM-SURVIVAL CELLS OF *SALMONELLA* TYPHIMURIUM ATCC 14028 AND *SALMONELLA* ENTERITIDIS PT 30 ON PAPER DISCS AND ALMONDS

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Fei Wang[4], Aubrey Mendonça[1,4]*, Byron Brehm-Stecher[1,4], James Dickson[2,4], Alan DiSpirito[3,4], and Angela Shaw[1]

[1]Department of Food Science and Human Nutrition, [2]Department of Animal Science, [3]Department of Biochemistry, Biophysics, and Molecular Biology, and [4]Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, Iowa 50011

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-2950. Fax: (515) 294-8181. Email: amendon@iastate.edu.

Key words: *Salmonella*, long-term-survival, desiccation, almonds

Abstract

The resistance exponential (EXP), stationary (STA) and long-term-survival (LTS) cells of *Salmonella* Typhimurium ATCC 14028 and *Salmonella* Enteritidis phage type PT30 to desiccation on paper disc and almonds was investigated. The LTS salmonellae were cultured in tryptic soy broth with 0.6% yeast extract (TSBYE) at 35 °C for 14 days. The EXP and STA cells cultured in TSBYE (35 °C) for 2.5 h and 24 h, respectively, served as control. The cells (10^7 CFU/mL) were air dried at 23 ± 1 °C on paper disc and almonds. Survivors were enumerated by plating diluted (10-fold) samples on tryptic soy agar with 0.6% yeast extract and counting bacterial colonies after incubation (35 °C, 24 h). The LTS cells were decreased in size with a coccoid shape and consistently exhibited the highest desiccation resistance ($P < 0.05$). After air drying for 24 hours on paper discs, reductions in initial viable counts of EXP, STA, and LTS *S.* Typhimurium were 6.34, 4.39 and 2.46 $\log_{10}$ CFU/mL, respectively; reductions in *S.* Enteritidis PT 30 were 6.5 (EXP), 3.75 (STA) and 2.62 (LTS). On almonds, reductions ($\log_{10}$ CFU/mL) of *S.* Typhimurium after air drying for 24 hours were 4.24 (EXP), 2.54 (STA) and 1.97 (LTS); reductions of *S.* Enteritidis PT 30 were 3.03 (EXP), 2.58 (STA) and 1.76 (LTS). Hot air (100°C) drying of almonds decreased *S.* Typhimurium STA and LTS cells by ~1.82 and 1.58 $\log_{10}$ CFU/mL, respectively, with a similar pattern of results observed for *S.* Enteritidis PT 30. The LTS state increases the resistance of *Salmonella* to desiccation thus contributing to enhanced survival of that pathogen during drying of almonds.

Introduction

Foodborne disease outbreaks and recalls involving dry foods contaminated with salmonellae have attracted much attention from federal and state regulatory agencies and the food industry [1-4]. For a Gram-negative human enteric pathogen *Salmonella* has an

unusual ability to survive for extended times in dry foods. Although *Salmonella* does not grow in low-water activity (low-a_w) foods, the long-term survival of this organism in chocolate, dried eggs, hard cheese, salami, and infant dried milk has been well documented [5]. From 2004 to 2011, seeds, and nuts products were the main low-a_w foods involved in recalls and market withdrawals in the USA and Canada associated with *Salmonella* [6]. The sesame product halva [7] has been associated with outbreaks of salmonellosis, along with desiccated coconut [8], a peanut butter coated savory snack [9, 10], peanut butter [11], peanuts [12], and raw almonds [2, 13].

Almonds could be naturally contaminated with *Salmonella* due to the nature of cultivation, collection, and epidemiologic history. Previous reports have shown the vast potential for long-term persistence of *Salmonella* Enteritidis PT 30 on almonds. *Salmonella* was isolated from an almond farm over a 5-year period, and all 53 obtained isolates were *Salmonella* Enteritidis PT 30, which were linked to two pulsed field gel electrophoresis patterns. This *Salmonella* strain was isolated from an outbreak in 2000 and 2001 that was associated with the consumption of raw almonds [14, 15]. During traceback investigations of a raw almond outbreak from 2000 to 2001, the outbreak strain, *S.* Enteritidis phage type (PT) 30, was isolated from almonds 8 months after collecting [2], indicating the long-term survival of the organism in this product. With the aim to reinforce the safety of almond consumption, the US Department of Agriculture and the Almond Board of California have mandated that all processed almonds in the US domestic market must be treated to reach a 4-log reduction of *Salmonella* enterica population [16, 17].

There are several varied repots on thermal resistance of *S.* Enteritidis on almond kernels. In one study a 5.47-log CFU/g reduction of the pathogen via dry heat treatment of almonds at 149 °C for 16 min was demonstrated [18]. Dry heat treatment (55- 60 °C for 4 days) of almonds inoculated with a four-strain mixture of *S.* Enteritidis resulted in a maximum inactivation of 1.30 $\log_{10}$ CFU/g [19]. Based on information from the Almond Board of California, a 4.0-log destruction of *S.* Enteritidis PT 30 can be achieved after 100 min of hot air treatment at 121°C [20]. Currently, the almond industry uses hot air roasting processes at temperatures ranging from 129.5 °C (265°F) to 154.5 °C (310°F) for 30–40 and minimum 10–15 min, respectively. All results of those previously stated research involved the use of the typically used overnight (18 to 24 h) cultures of *Salmonella* which largely represent cells in the stationary phase of their life cycle.

The stationary phase in bacteria is inevitably followed by the death phase and finally a long-term-survival (LTS) phase [21]. In the natural environment the LTS phase in bacteria is more the norm than the exception. Contrary to the notion that LTS cells may exist as a small su-population of cells, abundant forms of LTS cells have been reported in various bacterial species, such as *Micrococcus luteus* [22], *E. coli* [23] and *Listeria monocytogenes* [24]. The LTS phase was also observed in *S.* Typhimurium in our laboratory as viable counts of the pathogen were relatively stable at 6 to 7 $\log_{10}$ CFU/mL of TSBYE (35 °C) for over 22 months (unpublished data). The LTS phase cells of *L. monocytogenes* exhibited dramatically enhanced tolerance to heat and high pressure in comparison to stationary phase cells [24] and LTS phase cells of *S.* Typhimurium exhibited enhanced resistance to UV radiation compared to stationary and exponential phase cells (unpublished data). Based on those previously stated observations we

hypothesize that the LTS state can induce greater resistance of *Salmonella* to other physical food processes. To our knowledge there are no published reports on the resistance of LTS *Salmonella* to desiccation. Accordingly, the objective of the present study was to evaluate the desiccation resistance of LTS *Salmonella* Typhimurium and *Salmonella* Enteritidis PT 30 on paper discs and almonds kernels. The resistance of cells (in stationary and LTS phase) to hot-air drying on raw almonds was also evaluated.

Materials and Methods

Preparation of almonds

Raw almonds were obtained locally from a food store in Ames, Iowa. In this study, raw almonds, which have not been subjected to any pre-treatments, were used for desiccation tests. The background bacteria load, based on the aerobic plate count, was < 100 CFU/g and consisted largely of spore-forming bacteria based on colony morphology and microscopic examinations. The almonds were stored in sealed plastic bags and held at room temperature (23 ± 1 °C). The initial water activity of raw almonds was 0.545 ± 0.03, the average weight of raw almonds was 1.02 ± 0.08 g and average dimensions of the raw almond were 8.1 ± 0.5, 11.9 ± 0.6, and 23.1 ± 1.5 mm in thickness, width and length, respectively.

Microorganisms and culture conditions

Salmonella enterica serovar Typhimurium ATCC 14028 were obtained from the culture collection of the Microbial Food Safety Laboratory of Iowa State University. *Salmonella* Enteritidis PT 30 (ATCC BAA-1045), isolated from recalled 2000 to 2001 outbreak-associated almonds, was obtained from Thermo Fisher Scientific. Each stock culture in glycerol was maintained at -80°C and was transferred at least twice in 10 mL

tryptic soy broth with 0.6% yeast extract (TSBYE; Difco Laboratories, Detroit, MI), with incubation at 35°C for 24 h prior to use in experiments.

Exponential-, stationary- and long-term survival phases cells of S. Typhimurium and S. PT 30

Portions (1.0-mL) of *Salmonella* culture were transferred to TSBYE (100 mL) in a screw-capped 250 mL Erlenmeyer flasks. The inoculated medium was incubated at 35 °C with shaking at 150 rpm in a gyrorotary shaker incubator (New Brunswick Scientific Co. Inc., Edison, NJ) for 2.5 h, and 18 h to obtain exponential- and stationary-phase cells, respectively. *Salmonella* was cultured in TSBYE (100 mL) in a screw-capped 250 mL Erlenmeyer flask and incubated at 35 °C in incubator for 14 to 30 d to obtain long-term survival (LTS) cells.

Inoculum preparation

Exponential–phases cells from a 2.5-h culture, stationary-phase cells from an overnight (18 h) culture and LTS cells from 14 to 30 d culture in TSBYE (35°C) were harvested by centrifugation (Sorvall Super T21; Sorvall Product, L.P., Newtown, CT) at 10,000 ×g for 10 min at 22 ± 1 °C, washed once in saline to remove residual growth medium, then suspended in sterilized distilled water. The cell suspension of each of the three physiological states were adjusted to achieve 10^7 CFU/mL. The concentration of *S.* Typhimurium and *S.* PT 30 in the inoculum was determined by serial dilution in buffered peptone water (BPW, Difco), and plating onto both tryptic soy agar with 0.6% yeast extract (TSAYE; Difco) and xylose lysine tergitol agar (XLT4; Difco)

Transmission electron microscopy

The morphology and microstructure of stationary-phase (18 hours) and LTS (14 or 30 days) *Salmonella* Typhimurium cells were determined by transmission electron microscopy (TEM), All cells were harvested into 0.85% (w/v) saline at 23 ± 1 °C by centrifugation at 10,000 × g, 10 min, 4 °C. Aliquots (3-µl each) of cells in saline were placed onto a carbon film coated copper grid for 1 minute. The supernatant was removed by wicking from the side with a piece of filter paper and 3µl of 2% aqueous uranyl acetate was placed onto the grid for 30 seconds. The stain was removed by wicking and the grid was allowed to dry. Images were made using a JEOL 2100 scanning transmission electron microscope at an accelerating voltage of 200 kV (Japan Electron Optics Laboratory, USA, Peabody, MA).

Survival of desiccated bacteria on paper discs

The procedure was performed as described in a previous report with slight modifications [25]. Sterilized paper discs (6 mm in diameter; Remel, Lenexa, Kansas) were placed in sterile 100 × 15 mm petri dishes (Falcon, Tewksbury, MA). Each disc was inoculated with 20 µL of *Salmonella* cell suspension. The inoculated paper discs in the sterile petri dishes were air-dried in an incubator at 25 °C with plate lids off. The dried and inoculated paper discs were washed at 1, 2, 4, 8 and 24 h. Each paper disc was washed with 1 mL of BPW for at least 2 min. Serial dilutions of the cell suspension were prepared in BPW and 0.1 mL aliquots of appropriate dilutions were surface plated on TSAYE.

Almond inoculation

Experimental protocol was performed as described in a previous report [26]. Almond samples (400 ± 1 g) were weighed into polyethylene bags (Fisherbrand, 24oz;

Fisher), in which 25 ml of the inoculum was added. Populations on wet almonds were determined within an hour of inoculation by plating on TSAYE or were estimated by calculating the total population in 25 mL of inoculum and dividing by 400 g of almonds. Each bag was closed and inverted by hand for 1 min to thoroughly mix te almonds. The inoculated almonds were spread onto filter paper (Fisherbrand Qualitative P8, Fisher) on a metal drying rack. Almonds were dried for 24 h at 23 ± 1 °C. After 24 h, a weight of almonds sufficient for the study was placed into polyethylene zipper-top bags. The bags were sealed and placed into a larger polyethylene bag inside a plastic container, and stored at 4 °C for further use. Prior to each experiment, the containers were held at 23 ± 1 °C for 7 d before being held at the appropriate temperature to allow equilibration of the moisture added during inoculation.

Enumeration of inoculated almonds

Salmonella enterica serovar Typhimurium and *Salmonella* Enteritidis PT 30– inoculated almonds were removed from storage, and each bag was first inverted by hand for 20 s to mix thoroughly. Ten almond kernels were placed into a 532-mL Whirl-Pak bag with 10 mL of BPW and pummeled in a laboratory Stomacher blender for 2 min (Stomacher 80 Biomaster Lab Blender, Seward, Thetford, UK). Three recovery methods including hand shaking, mechanical shaking and stomaching were compared for preparing almond samples previously. There is a small but significant ($P < 0.05$) improvement in recovery of *Salmonella* from almonds was observed with stomaching [26]. So the stomaching method was used to recover *Salmonella* from almonds. Serial dilutions of the cell suspension were prepared in BPW, and 0.1 mL aliquots of appropriate dilutions were surface plated on TSAYE.

Hot air treatment of inoculated almonds

Hot air treatment was applied as described previously [27]. Inoculated almonds were removed from storage at 4 °C and tempered to 23 ± 1 °C over a 2- to 3-h period. Almonds (25 g) were arranged in a single layer on aluminum screen trays (ca. 11 cm in diameter by 2 cm in height) and placed in a forced-air Fisher Scientific Isotemp oven (100 ±0.25 °C; model 851F, Fisher Scientific, Dubuque, IA). Almonds (25 g samples) were exposed to forced air at 100 °C for 10, 15, 20, 25 and 30 min. After heat treatments, almonds were immediately placed in a Stomacher 400 bag (Seward Medical Ltd., London, UK) containing 100 mL of BPW and pummeled for 2 min at normal speed. Serial dilutions of the cell suspension were prepared in BPW, and 0.1 mL aliquots of appropriate dilutions were surface plated on TSAYE.

Statistical analysis

Three replications of each experiment were performed. Mean numbers of pathogen survivors were analyzed using SAS statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Significant differences were defined at $P < 0.05$ for all the experimental data.

Results

Transmission electron microscopy

Representative electron micrographs of stationary (18 h) and LTS (14 days) cells of *S.* Typhimurium ATCC 14028 are shown in Figure 1. Transmission electron microscopy results reveal that both exponential phase cells (data not shown) and stationary phase cells were rod shaped; however, as the pathogen transitioned into the long-term-survival state it became smaller in size (decrease in cell length) and transformed into a coccoid shape. In most instances the cytoplasm of the LTS cells

appeared shrunken and condensed with prominent clear areas that were not present in the stationary phase cells.

Desiccation resistance on paper discs in room temperature

Desiccation resistance based on log CFU/mL reduction of viable counts of three physiological states of *S.* Typhimurium or *S.* Enteritidis PT30 on paper discs at ambient temperature (23 ± 1 °C) is shown in Figure 2. The initial viable count for each growth phase of the pathogens were ~ 6 $\log_{10}$ CFU/mL. The reduction in numbers of survivors for both *S.* Typhimurium and *S.* PT30 increased with the increase of drying time. Reductions of exponential cells for *S.* Typhimurium or *S.* Enteritidis PT30 reached up to 6 $\log_{10}$ CFU/mL after 2-4 hours of drying. For stationary cells of *S.* Typhimurium and *S.* Enteritidis PT30, reductions ($\log_{10}$ CFU/mL) were 4.39 and 3.76, respectively, after 24 h of drying. In contrast, viability of LTS. Typhimurium and *S.* PT30 declined by only 2.46 and 2.62, respectively, after 24 h. These results clearly indicate that the LTS cells of *S.* Typhimurium and *S.* PT30 have a better ability to survive the lethal effect of desiccation in ambient temperature in comparison to exponential- or stationary- phase cells.

Desiccation resistance on raw almonds in room temperature

Reductions (log CFU/mL) of *S.* Typhimurium and *S.* Enteritidis PT30 from desiccation on raw almonds at 23 ± 1 °C are shown in Figure 3. Reductions of LTS cells for both pathogens on raw almonds were lower compared to exponential- and stationary-phase cells. Populations of the pathogens on raw almonds decreased with increasing drying time. For *S.* Typhimurium, reductions (log CFU/mL) in exponential- and stationary-phase cells were 4.24 and 2.54, respectively after 24 h of drying at 23 ± 1 °C, whereas LTS cells declined only 1.97 log CFU/mL (Fig. 2A). Similarly, for *S.* Enteritidis PT30, reductions (log CFU/mL) were 3.02 (exponential), 2.59 (stationary) and

1.76 (LTS). These results indicte that LTS cells, compared to exponential- or stationary cells, have a greater capability to resist the lethal effects of desiccation on raw almonds at 23 ± 1 °C.

Desiccation resistance under hot air treatment of almonds

Reductions from thermal inactivation of *S.* Typhimurium and *S.* Enteritidis PT30 on raw almonds heated at 100 °C for 30 min are shown in Figure 4. Exponential cells were significantly sensitive to desiccation even at 23 ± 1 °C; therefore, only stationary and LTS cells were used for the hot air treatment. Initial viable counts of stationary cells of *S.* Typhimurium and *S.* Enteritidis PT30 decreased by 2.02 and 2.28 log $_{10}$CFU/mL, respectively following air heating of almonds at 100 °C for 30 min. The LTS cells of *Salmonella* were more resistant ($P < 0.05$); after a 30 min-heating at 100 °C, initial viable counts (log $_{10}$CFU/mL) of LTS cells decreased by only 1.88 (*S.* Typhimurium) and 1.85 (*S.* Enteritidis PT30).

Discussion

Salmonella is able to adapt to extreme environmental conditions such as lower or higher than optimal temperatures, pH values, or desiccation [28] and may survive for months or even years in certain low moisture foods. In this study, the LTS cells of *S.* Typhimurium and *S.* PT30 exhibited significantly ($P < 0.05$) greater capability than exponential- or stationary-phase cells to endure desiccation conditions on paper discs and almonds. This resistance to desiccation that is significantly higher than that of stationary cells may arise from of cellular changes as the pathogen transitions into the LTS phase. The LTS phase in bacteria may result in cell dormancy whereby growth is temporarily halted and metabolic activity is minimized to conserve energy. Loss of functional rRNA and downregulation of ribosomal protein genes during the LTS phase may lead to lower

protein translation and subsequent dormancy. Dormancy may therefore be viewed as an adaptive strategy to cope with suboptimal growth conditions and enhance the long-term survival of bacteria [48]. We obsrved that transfer of 42-day old LTS *Salmonella* to fresh sterile TSBYE (35 °C) resulted in the coccoid cells reverting to rod shaped cells. Further research is needed to determine if LTS S. Typhimurium (Figure 1 B) is in a dormant state.

We hypothesize that the enhanced desiccation resistance observed in LTS S. Tphimurium in the present study is due to condensation of the cytoplasm. As a consequence, water activity is lowered in the coccoid cells compared to the stationary or exponential phase *S.* Typhimurium cells. Compared to exponential cells, LTS-cells possess much lower transcription activities, which indicate metabolic dormancy [29]. Wen et al. reported *Listeria monocytogenes* in LTS phase are more resistant than exponential- or stationary-phase cells to high pressure and heat [24]. Those authors also speculated that the enhanced barotolerance in the LTS phase is presumably due to cytoplasmic condensation and subsequent lowered water activity in coccoid *L. monocytogenes* cells.

Another explanation for the enhanced desiccation resistance observed in S. Typhimurium might be the intracellular accumulation of solutes. For instance, *L. monocytogenes* takes up high levels of compatible solutes from the growth medium and accumulated them the cytoplasm during the LTS phase, leading to the enhanced resistance to various environmental stresses [29]. A major compatible solute is trehalose and a variety of stress conditions can cause its accumulation in bacteria. This accumulate of trehalose can protect cells against stresses, such as heat, cold, oxidation and

desiccation [30]. Also, high concentrations of trehalose also resulted in lowered intracellular water activity [31]. By stabilizing proteins, cells surviving with lowered water activity may possess better resistance against high temperature [32].

Based on the results of the present study, *Salmonella* cells transformed to a coccoid shape and exhibited significantly ($P < 0.05$) enhanced desiccation resistance compared to stationary and exponential phase cells. When these coccoid cells were placed in fresh sterile growth medium they changed back into rods with a consequent lowering of desiccation resistance to the level of the stationary phase cells after 18 h of incubation (data not shown). These findings warrant evaluation of other *Salmonella* serovars to confirm morphological changes in the LTS phase and resistance to desiccation and heat. The use of stationary phase pathogens in food safety research is highly prevalent and almost scientific dogma based on the belief that stationary phase cells might be the most resistant. Results of the present study have demonstrated that this belief is not true at least with respect to *S*. Typhimurium ATCC 14028 and S. Enteritidis PT30. Additionaly, other researchers have demonstrated the higher pressure and heat resistance of LTS cells of *L. monocytogenes* compared to stationary phase cells of that pathogen [24]. Taken together, these findings suggest that the development of pathogen control methods based on the use of stationary phase cells is likely to overestimate the effectiveness of an antimicrobial intervention. Also, salmonellae could be present as LTS cells in biofilms and hard to-clean areas and transform into active rod shaped cells upon contamination of ready-to-eat foods after processing or preservation.

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Figures

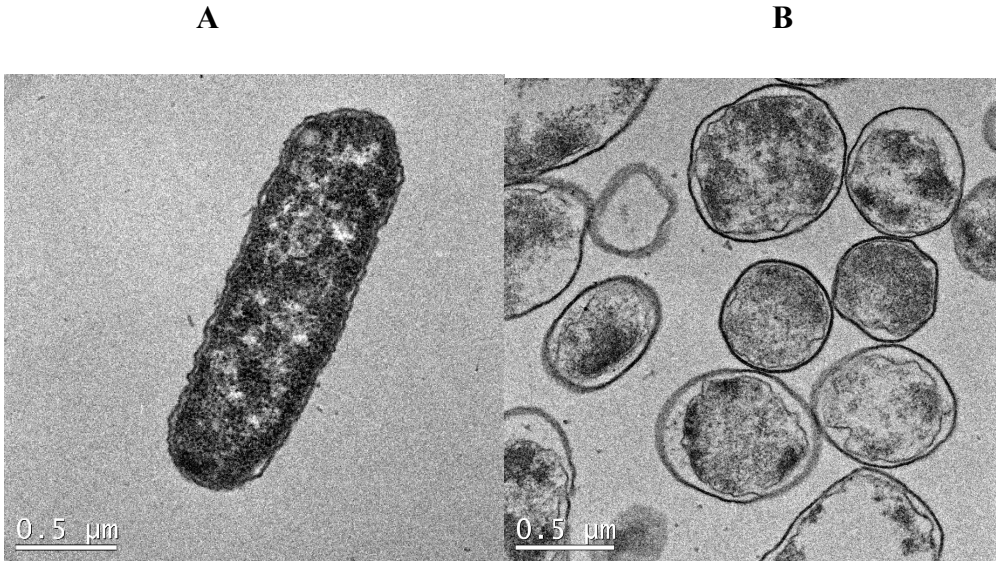
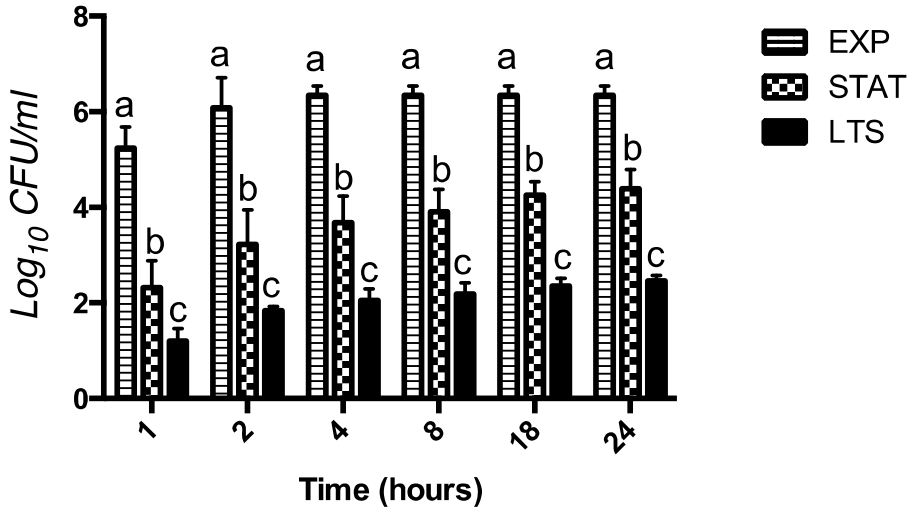


Figure 1. Transmission electron microscopy images of *Salmonella* Typhimurium ATCC 14028 at stationary phase (**A**) and long-term-survival (LTS) phase (**B**)

A



B

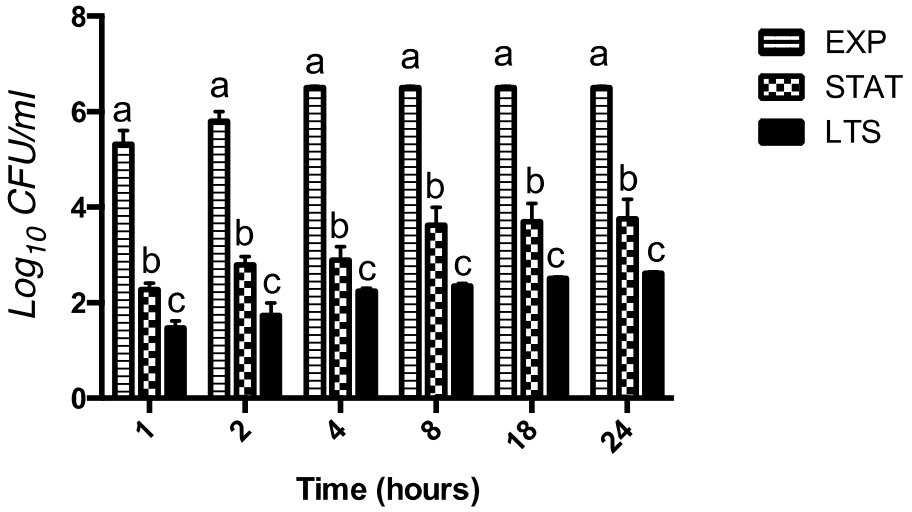
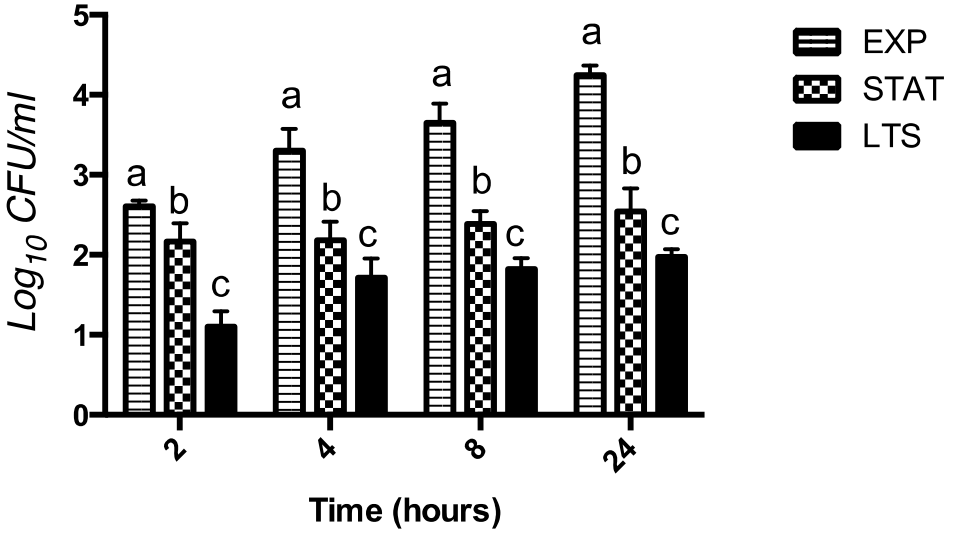


Figure 2. Log reduction of exponential, stationary and LTS *S.* Typhimurium (A) and *S.* Enteritidis PT 30 (B) following air dry on paper discs at ambient temperature (~23 ± 1 °C). Data represent mean values ± SDs from three replicate experiments. For each exposure time point, bars that do not share a common letter are significantly different ($p<0.05$).

A



B

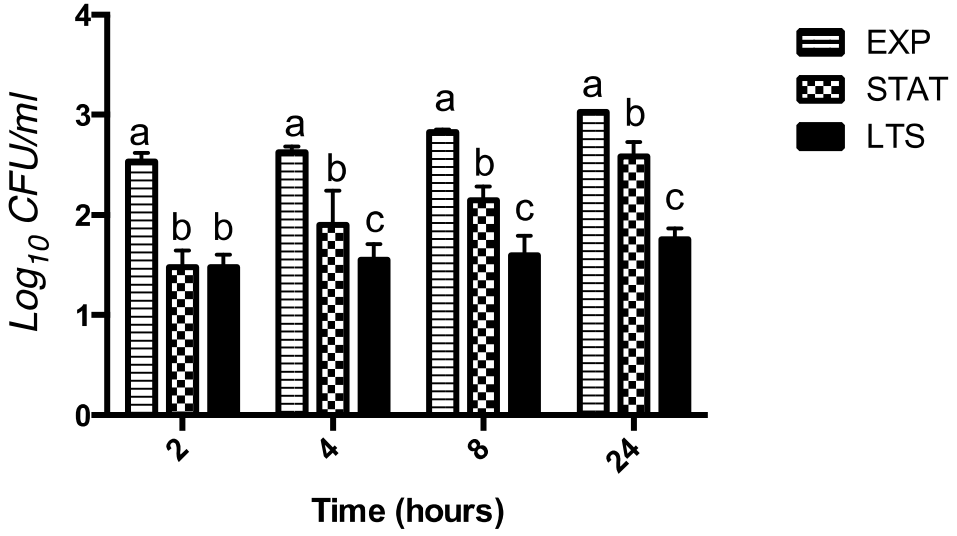
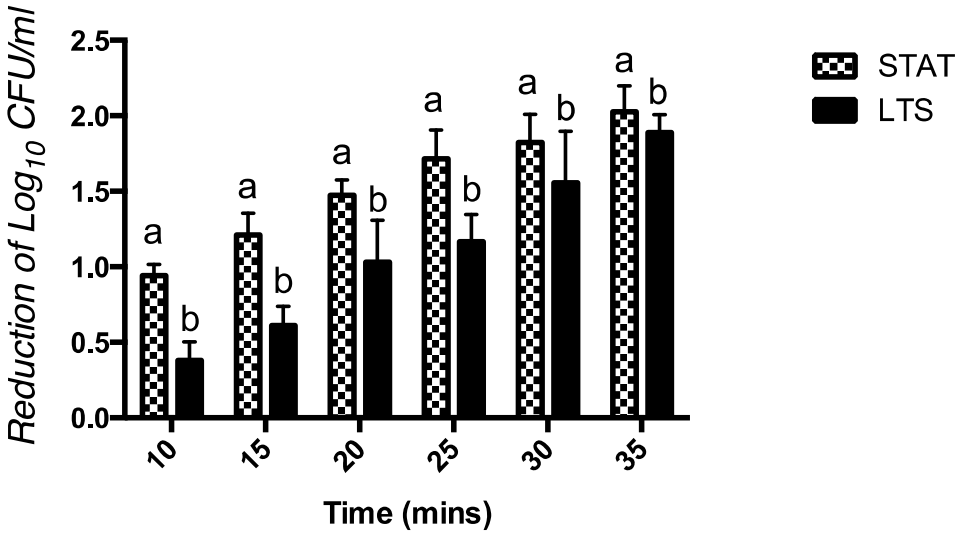


Figure 3. Log reduction of exponential, stationary and LTS *S.* Typhimurium (A) and *S.* Enteritidis PT 30 (B) following air dry on raw almonds at ambient temperature (~23 ± 1 °C). Data represent mean values ± SDs from three replicate experiments. For each exposure time point, bars that do not share a common letter are significantly different ($p<0.05$).

A



B

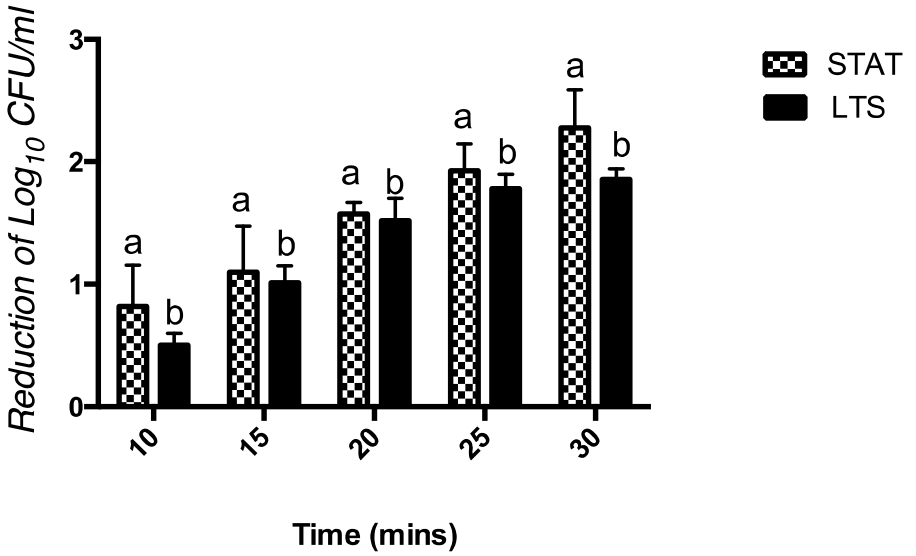


Figure 4. Log reduction of exponential, stationary and LTS *S.* Typhimurium (A) and *S.* Enteritidis PT 30 (B) following heat dry on raw almonds at 100°C. Data represent mean values ± SDs from three replicate experiments. For each exposure time point, bars that do not share a common letter are significantly different ($p<0.05$).

CHAPTER 5 ANTIMICROBIAL EFFICACY OF CINAMALDEHYDE AGAINST STATIONARY PHASE CELLS AND LONG-TERM-SURVIVAL PHASE CELLS OF *ESCHERICHIA COLI* O157:H7 IN CARROT JUICE AND APPLE JUICE

A paper to be submitted to Journal of Food Protection

Fei Wang[4], Aubrey Mendonça[1,4]*, Byron Brehm-Stecher[1,4], Aura Daraba[1,5], James Dickson[2,4], Alan DiSpirito[3,4], and Angela Shaw[1]

[1]Department of Food Science and Human Nutrition, [2]Department of Animal Science, [3]Department of Biochemistry, Biophysics, and Molecular Biology, and [4]Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, Iowa 50011; [5]Department of Food Science, Food Engineering and Applied Biotechnology, University "Dunarea de Jos" of Galati, Romania

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food

Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-2950. Fax: (515) 294-8181. Email: amendon@iastate.edu.

Key words: *Escherichia coli* O157:H7, long-term-survival, cinnamaldehyde, juice

Abstract

The efficacy of cinnamaldehyde for inactivating stationary (STA) and long-term-survival (LTS) cells of *Escherichia coli* O157:H7 in carrot juice and apple juice was investigated. Peptone water, pH-adjusted peptone water (PAPW), carrot juice and apple juice with added cinnamaldehyde ranging from 0.25 to 1.5 µL/mL, were used in the present study. LTS and STA *E. coli* O157:H7 were cultured in tryptic soy broth with 0.6% yeast extract at 35 °C for 14 days and 20-24 h, respectively. All solutions were inoculated with STA or LTS cells to obtain 6.01 $\log_{10}$ CFU/mL. Inoculated peptone water or juices without cinnamaldehyde were used as controls. The *E. coli* survivors were enumerated by plating diluted (10-fold) samples on tryptic soy agar with 0.6% yeast extract and counting bacterial colonies after incubation (35°C, 24 h). LTS cells exhibited the highest resistance to cinnamaldehyde in peptone water and juices ($P < 0.05$). Cinnamaldehyde (1.5 µL/mL) totally inactivated STA *E. coli* O157:H7 (negative by enrichment) after 72 h in peptone water and carrot juice whereas, LTS cells ($\log_{10}$ CFU/mL) were 1.61 and 2.35 in peptone water and carrot juice, respectively. Cinnamaldehyde (1.5 µL/mL) eliminated STA cells of *E. coli* in PAPW and apple juice after 8 h; however, LTS cells ($\log_{10}$ CFU/mL) remained at 2.80 (in PAPW) and 2.26 (in apple juice). *E. coli* O157:H7 LTS cells are more resistant to cinnamaldehyde than STA cells and should be used in studies as a conservative approach to validate the antibacterial efficacy of cinnamaldehyde against *E. coli* in juices.

Introduction

Outbreaks of illness resulted from bacteria and parasites have been linked to juices and ciders for quite a long time [1-3]. From 1923 to 2000, at least 28 foodborne illness outbreaks have implicated contaminated fruit juices [4]. Enteric foodborne

pathogens such as *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium, parasitic protozoa including *Cryptosporidium parvum*, and Gram-positive pathogens like *Listeria monocytogenes* have all been reported to survive in raw fruit and vegetable juices [5-8]. Microorganisms such as *E. coli* O157:H7 and *Salmonella* spp. are associated with fruit juice outbreaks in the U.S. [3, 4, 9]. Commercially available fruit and vegetable juices are typically heat pasteurized, ultra-high temperature processed, or canned to guarantee microbiological safety and extended shelf-life. Despite of the fact that these processes are effective in eliminating pathogenic microorganisms, they negatively affect quality of juices such as flavor, color, and nutrition [10]. To respond to consumers' demand for minimally processed foods with fresh-like attributes and increasing public concern over perceived health risks from synthetic food preservatives, juice manufacturers are continuously seeking alternatives to traditional physical or chemical processes to enhance the microbial safety of juices and newly developed juice blends. One promising alternative is to use plant essential oils and their isolated antimicrobial components [11, 12].

Cinnamaldehyde makes up1–8%, 55–75%, and 70–95% of the essential oil from cinnamon leaf, cinnamon bark, and cassia cinnamon bark, respectively [13]. Generally, cinnamaldehyde is considered as safe by the U.S. Food and Drug Administration and is approved for food use (21 CFR 182.60). Based on the U.S. Flavoring Extract Manufacturers Association, toxicity studies (sub chronic and chronic) demonstrated that cinnamaldehyde has a broad margin of safety [14]. Its antimicrobial activity may be due to its lipophilic group penetrating the membrane to the interior of the cell to exert inhibitory activity at the target site [15]. Furthermore, the antioxidant activity of

cinnamaldehyde may diminish dehydration and occurrence of browned polymers that are responsible for the mushroom browning and shriveling [16, 17]; it has been studied in the preservation of a great variety of foods, including fruit, vegetables, fish, dairy products, and meat [18]. Cinnamaldehyde is an effective natural antimicrobial that can be used to inactivate bacterial pathogens in fruit and vegetable juices to enhance microbial safety of these food products [19]. Although cinnamaldehyde has antimicrobial activity [18, 20, 21], its effectiveness in enhancing the microbial safety of juices needs to be validated.

As claimed by the traditional microbiology textbooks, the bacterial life cycle consists of three or four phases[22]. However, from a practical perspective, actually five phases exist， namely, lag phase, exponential or logarithmic phase, stationary (STA) phase, death phase and extended or long-term-survival (LTS) phase [22, 23], which is a period of prolonged survival [23]. Aging broth cultures of *Sarcina lutea* and *Serratia marcescens* had a "senescent phase" to remain alive within a two-year incubation[23]. *E. coli* can be retained for a long time without the addition of nutrients[23-25] in a “long-term stationary phase”[22]. The LTS phase was observed in *L. monocytogenes* to have high viable cells density at 10^8 CFU/mL in tryptic soy broth with yeast extract (TSBYE) for over 1 month[26]. The LTS state in *L. monocytogenes* enhanced that pathogen’s resistance to heat and high pressure compared to exponential- and STA-phase cells [26], thus challenging the antimicrobial effectiveness of food processing and preservation methods.

To date, no systematic investigation has been made on the LTS phase of *E. coli* O157:H7 with regard to the pathogen’s resistance to natural antimicrobials such as cinnamaldehyde. *E. coli* O157:H7 has a low infectious dose; therefore, the LTS state

could potentially pose a serious food safety threat should it permit even low numbers of the pathogen to survive with enhanced resistance to antimicrobials. The present work aims to explore the influence of LTS on the fate of *E. coli* O157:H7 exposed to cinnamaldehyde in non-acidic system, peptone water and carrot juice, as well as acidic system, pH-adjusted peptone water and apple juice.

Materials and Methods

Microorganisms and culture conditions

E. coli O157:H7 ATCC 35150 were used in this study and obtained from the culture collection of the Microbial Food Safety Laboratory of Iowa State University. Each glycerol stock was maintained at -80 °C and was transferred at least twice in 10 mL of tryptic soy broth with 0.6% yeast extract (TSBYE; Difco Laboratories, Detroit, MI), with incubation at 35°C for 24 h prior to each experiment.

Preparation of exponential-, stationary- and long-term survival phases cells of E. coli

A portion (1.0 mL) of *E. coli* O157:H7 ATCC 35150 culture was transferred to TSBYE (100 mL) in a screw-capped 250 mL Erlenmeyer flask. The inoculated medium was incubated at 35 °C with shaking at 150 rpm in a gyrorotary shaker incubator (New Brunswick Scientific Co. Inc., Edison, NJ) for 3 h, and 18 h to obtain exponential-, and stationary-phases cells, respectively. *E. coli* was cultured in TSBYE (100 mL) in a screw-capped 250 mL Erlenmeyer flask and incubated at 35 °C in incubator for 15-30 days to obtain long-term-survival (LTS) cells. Cells in a 30-mL culture of *E. coli* O157:H7 were harvested by centrifugation (10,000 × g, 10 min, 4 °C) by using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT), and washed once in 0.85% (w/v) saline. Pelleted cells were suspended in fresh saline to obtain a final viable cell concentration of 9.0 $\log_{10}$ colony-forming units (CFU/mL). Colony counts of the

washed cell suspensions were evaluated by serially diluting (10-fold) and surface plating samples on tryptic soy agar (Difco; Becton Dickinson) supplemented with 0.6% yeast extract (TSAYE) followed by counting bacterial colonies on TSAYE after incubation (35°C) for 24 h.

Preparation and inoculation of test samples

Commercially available pasteurized carrot juice and apple juice without added preservatives, and each juice from the same production lot, were purchased from a local grocery store. The level of background microflora in the apple juice and carrot juice were determined by surface-plating serial dilutions (0.1% peptone) on TSAYE. The pH-adjusted peptone water was prepared by adjusting the pH to 3.13 with 1M aqueous HCl solution. The juices and peptone were transferred in a portable cooler to the laboratory and stored at 4 ± 0.2 °C until they were used. Specifically, 1.0-mL aliquots of each juice and peptone water were added to 1.0-mL portions of sterile buffered peptone water (BPW; Becton Dickinson) at pH 7.2 ± 0.2. The entire 2.0-mL mixture was surface plated on 8 plates (0.25 mL per plate) of TSAYE. Bacterial colonies were counted after 24 h of incubation (35 °C). In addition, 1.0-mL aliquots of each juice and peptone water were added to tubes of sterile TSBYE (10 mL each), followed by incubation (35 °C) for 24 h. After incubation, looped samples of TSBYE were streak-plated on appropriate selective agar, which was then incubated (35 °C) and examined for bacterial colonies at 24 and 48 h. A portion (400 mL) of each juice or peptone was aseptically transferred into a separate sterile screw cap glass bottle and held at 4 °C. Based on the study from Manu et al., the minimum inhibitory concentration of cinnamaldehyde for *E. coli* O157:H7 in BHI was 0.25 µL/mL [19]. In this study, filter-sterilized cinnamaldehyde was added to each type of juice and peptone water to give the following concentrations: 0 (control), 0.25, 0.5,

1.0, and 1.5 µL/mL. Bottles of juice and peptone water were capped, vigorously shaken, and inoculated with 4.0mL of a diluted (1:100 in 0.85% saline) cell suspension of *E. coli* O157:H7 to give a final cell concentration of 6.01 $\log_{10}$ CFU/mL for each pathogen. Each bottle of juice and peptone water was swirled to mix its contents and stored at 4 °C.

Measurement of pH and degrees BRIX

Measurements of pH were taken by using an Orion Model 525 pH meter (Orion Research, Inc., Boston, MA) that was fitted with a glass electrode. Before measuring the pH, all juice samples were tempered to 23 ± 1 °C. A digital Pocket Refractometer PAL (ATAGO, USA, Inc., Bellevue, WA) was used to take degrees BRIX measurements.

Microbiological analysis

Inoculated carrot juice and peptone water stored at 4 °C were tested for viable pathogens at 2, 4, 8, 24, 48 and 72 h. Inoculated apple juice and pH-adjusted peptone water stored at 4 °C were tested for viable pathogens at 1, 2, 4, 8, 24 and 48 h. Ten-fold serial dilutions of the juices and peptone water were prepared by using sterile BPW (pH 7.2). Aliquots (1.0 or 0.1 mL) of juice were surface plated (in duplicate) on TSAYE. Agar plates were incubated at 35 °C, and bacterial colonies were counted at 24 h. All agar media were purchased from Difco (Becton Dickinson). The inoculated juices and peptone water were enriched by aseptically transferring them to an enrichment broth and incubation (35 °C) for 48 h. Looped samples of enrichment broth were streak plated on TSAYE and incubated (35 °C) for 24 h.

Statistical analysis

Three replications of each experiment were performed. Mean numbers of pathogen survivors were analyzed using SAS statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Significant differences were defined at $P < 0.05$ for all the experimental data.

Results

Cell viability

The growth curve of *E. coli* O157:H7 ATCC 35150 inoculated in TSBYE at 35°C is shown in Figure 1. The cells were in log phase (A in Fig.1) for 0.75 day (18 h). The population reached at 9.40 $\log_{10}$CFU/mL and remained for ~24 h in the STA phase (B in Fig.1). After 2 days, the cells entered death phase (C in Fig.1) and the viable population dropped significantly to 8.02 log CFU/mL, corresponding to a ~90% loss of cells viability. Nevertheless, the death of cells eventually ceased and the viable counts remained relative stable at 5 to 6 $\log_{10}$ CFU/mL for 20 days without any additional nutrients supplied. We named this period the long-term-survival phase (D in Fig.1).

Juice characteristics and initial viable count

For Carrot juice the initial average pH and degrees Brix (°BRIX) were 6.26 and 8.1, respectively. The initial average pH and °BRIX of apple juice were 3.63 and 6.25, respectively. The average pH of regular peptone water was 6.89 and 3.13 for the pH-adjusted peptone water. The addition of cinnamaldehyde did not significantly alter the pH or °BRIX in the juices or peptone solutions (data not shown). The average initial viable count for *E. coli* O157:H7 in artificially inoculated control juice and juice with cinnamaldehyde was 6.01 (± 0.2) $\log_{10}$ CFU/mL.

Viability of pathogens in peptone water and carrot juice

Viable counts of STA and LTS phase cells of *E. coli* O157:H7 in control peptone water after 72 h were 5.09 and 5.19 $\log_{10}$ CFU/mL, respectively (Table 1). For STA cells, significant ($p < 0.05$) decrease of the pathogen first occurred at 24 and 8 h in peptone water containing cinnamaldehyde at 0.5, 1.0 and 1.5 µL/mL, respectively. (Table 1A). For LTS phase cells, significant ($p < 0.05$) decrease occurred after 48 h and 24 h in juice with cinnamaldehyde at 0.5, 1.0, or 1.5 µL/mL (Table 1B), indicating greater sensitivity of stationary-phase cells to cinnamaldehyde in peptone water. For LTS-phase cells, no complete inactivation (based on enrichment tests) of *E. coli* O157:H7 was observed in peptone water with various cinnamaldehyde concentrations tested (Table 1A). In contrast, for stationary-phase cells, cinnamaldehyde (1.5 µL/mL) completely inactivated *E. coli* O157:H7 after 72 h of storage (Table 1B) according to results of selective plating and enrichment. Increased pathogen sensitivity to cinnamaldehyde for STA-phase cells over LTS phase cells of *E. coli* was also observed in carrot juice (Table 2A, B). At 72 h, cinnamaldehyde (1.5 µL/mL) completely inactivated (negative enrichment test) STA-phase cells of *E. coli* O157:H7 in carrot juice while 2.35 $\log_{10}$ CFU of LTS cells per mL remained (Table 2A, B).

Viability of pathogens in pH-adjusted peptone water and apple juice

As shown in Table 3, reductions of STA and LTS phase cells of *E. coli* O157:H7 exposed to the PAPW (pH 3.13) with cinnamaldehyde (1.5 µL/mL) after 1 h were 1.20 and 0.2 $\log_{10}$ CFU/mL, respectively. As for STA-phase cells, cinnamaldehyde at 1.0 and 1.5 µL/mL completely inactivated *E. coli* O157:H7 in pH-adjusted peptone water after 24 and 8 h and no STA-phase cells were detected by plating or enrichment. In contrast, viable LTS cells of *E. coli* O157:H7 were 2.69 and 2.80 $\log_{10}$ CFU/mL, in pH-adjusted

peptone water with 1.0 and 1.5 μL/mL cinnamaldehyde after 24 and 8 h, respectively. Taken together, the survival of LTS phase cells of *E. coli* O157:H7 in the more acidic peptone water was greater than that of STA-phase cells (Table 3).

Viable counts of STA-phase and LTS phase cells of *E. coli* O157:H7 after 1 h of exposure to apple juice with cinnamaldehyde (1.5 μL/mL) were only 4.45 and 5.22 $\log_{10}$ CFU/mL, respectively (Table 4). The STA-phase cells of *E. coli* O157:H7 were not detected (by plating or enrichment) after 24 and 8 h in apple juice with 1.0 and 1.5 μL/mL of cinnamaldehyde. In contrast, viable counts of LTS cells of *E. coli* O157:H7 were 2.63 and 2.26 log10 CFU/mL in apple juice with 1.0 and 1.5 μL/mL cinnamaldehyde after 24 and 8 h, respectively. On the basis of forgoing results, LTS phase cells of *E. coli* O157:H7 clearly showed stronger resistance to cinnamaldehyde in apple juice than STA-phase cells.

Discussion

Pathogens in most juices can be killed by thermal methods like pasteurization, which might change the composition and flavor properties of the juices [27], or non-thermal methods such as using preservatives such as potassium sorbate and sodium benzoate, which show minimal lethal effects against pathogens[28]. Due to the concerns of negative effects of in processed foods, customers prefer natural additives and preservations [29], such as herb extract [30], caffeic acid [31] and nisin and cinnamon [15]. Nearly all the published reports on inactivation effects of essential oils against pathogens used STA-phase cells and there has been no effort comparing the resistance to essential oils between STA-phase cells and LTS phase cells. The LTS phase cells of *L. monocytogenes* exhibited more resistance than STA-phase cells against high temperature and pressure [26]. As a result, the present study investigated the potential of

cinnamaldehyde to inactivate STA-phase cells and LTS phase cells of *E. coli* O157:H7 in peptone water and juices with different pH. Given that the pH of carrot juice is 6.26, regular peptone water whose pH is 6.89 could serve as the comparable model system. Apple juice has a pH of 3.63 and the comparable model system is pH-adjusted peptone water with pH 3.13.

Inactivation of *E. coli* O157:H7 in peptone water and two juices at different pH was dependent on the concentration of cinnamaldehyde. For example, the viable counts for LTS phase cells of *E. coli* O157:H7 in carrot juice with cinnamaldehyde at 0.25, 0.5 1.0 and 1.5 µL/mL were 5.56, 5.08, 4.45 and 3.89 $\log_{10}$ CFU/mL in 48 h, respectively (Table 2B). Identically, in apple juice (pH 3.63) with same concentrations of cinnamaldehyde, viable counts for LTS phase cells were 4.87, 2.78, 1.81 and negative by enrichment, respectively (Table 4B). It is clear that the antimicrobial effect of cinnamaldehyde is improved by low pH. While this pH-enhanced antibacterial effect resulted in elimination of STA-phase cells (negative enrichment) as early as 8 h by 1.5 µL/mL cinnamaldehyde (Table 4A), at that same time and cinnamaldehyde concentration, LTS cells at 2.26 log10 CFU/mL remained in the juice, indicating that LTS cells have a far higher tolerance for cinnamaldehyde compared to STA cells. In fact, at 8 h 1.5 µL/mL cinnamaldehyde decreased the viable count of STA cells by ~6.0 log CFU/mL whereas, LTS cells were decreased by only 3.75 log in apple juice. Considering the juice HACCP regulation for juice processors to demonstrate a 5 log reduction in juice of the pertinent pathogen, the apple juice in the present study would be in compliance base on results from STA cells (6 log reduction) but not LTS cells (3.75 log reduction). These results the physiological state such as LTS phase of *E. coli* O157:H7 can have

significantly different (P<0.05) resistances against the antibacterial effect of cinnamaldehyde.

Similar trend in results were observed in the non-acidic liquid media used in the present study. In peptone water with 1.5 µL/mL cinnamaldehyde, STA phase cells of *E. coli* O157:H7 counts were lowered to undetectable level, which is over 6 $\log_{10}$ CFU/mL, after 72 h (Table 1A). However, the identical cinnamaldehyde treatment only caused less (4.4 $\log_{10}$ CFU/mL reduction) for LTS phase cells after 72 h (Table 1B). Similarity, over 6 $\log_{10}$ CFU/mL of stationary phase cells of *E. coli* O157:H7 were inactivated by 1.5 µL/mL of cinnamaldehyde in carrot juice after 72 h (Table 2A). In contrast, only 3.66 $\log_{10}$ CFU/mL reduction of LTS phase cells was observed in the same concentration of cinnamaldehyde in carrot juice after 72 h (Table 2B).

The higher resistance of LTS phase cells to antimicrobials might be related to th Toxin/antitoxin (TA) system [32]. Ectopic expression of *RelE* and *MazF* strongly enhanced the tolerance to antibiotics [33, 34]. *RelE* and the antitoxin gene *mazE* and toxin gene *mazF* were dramatically up-regulated (over 100-fold increase in mRNA levels) when *Listeria. monocytogenes* entered into LTS phase [35]. Dormancy could be another possible reason to enhance LTS cells in the presence of environmental stress, such as antimicrobial effects from cinnamaldehyde. Within LTS phase, cells may stay dormant due to the low synthesis activity of protein [35].

The pH of the solutions caused significant effects on the antibacterial activity of cinnamaldehyde on *E. coli* O157:H7 counts. For instance, complete inactivation of STA phase cells of *E. coli* O157:H7 was observed in pH-adjusted peptone water (pH 3.13) with 1.0 µL/mL cinnamaldehyde in 24 h (Table 3A). However, there were 3.15 $\log_{10}$

CFU/mL of LTS phase cells of *E. coli* O157:H7 alive under the same concentration of cinnamaldehyde and treatment time in regular peptone water (pH 6.89) (Table 1A). Manu et al. reported that the low pH (3.39) of mixed berry juice positively affected the antibacterial activity of cinnamaldehyde by causing acid-induced injury for *E. coli* O157:H7 and *Salmonella* Typhimurium. Our study demonstrated that *E. coli* O157:H7 survived better in high pH solution with added cinnamaldehyde. Since the main target of cinnamaldehyde is the cytoplasmic membrane [36], the low pH could denature membrane proteins and/or increase the hydrophobicity of cinnamaldehyde thus permitting greater interaction with the bacteria membrane. Therefore, the acidity of mixed berry juice most likely increased the sensitivity of pathogen to the membrane-damaging effect of cinnamaldehyde.

Cinnamaldehyde may also inhibit ATPase activity of *Escherichia coli* at sub-lethal concentration [37]. ATPase located on cytoplasmic membrane [38] which can be inhibited by cinnamaldehyde across the outer membrane of Gram-negative bacteria [37]. Despite the inconclusive mechanism of the antibacterial effects of cinnamaldehyde, it is assumed that cinnamaldehyde inactivates bacteria by inhibiting ATPase at sub-lethal concentrations and disrupting the cytoplasmic membrane at lethal concentrations [37]. Considering the possible cellular sites or functions where cinnamaldehyde might exert its antimicrobial effect, the observed tolerance of LTS phase *E. coli* O157:H7 to cinnamaldehyde might be associated with alterations of target sites or metabolic function(s) that occur when bacter transition into the LTS phase.

Conclusions

The LTS phase cells show enhanced resistance to cinnamaldehyde as compared with STA phase cells of *E. coli* O157:H7, the most commonly used physiological state of

cells in previous studies. The Food and Drug Administration (FDA) has requested that the most resistant cells must be used in challenge studies. Therefore, LTS phase cells should be used rather than STA phase cells to evaluate the effectiveness of cinnamaldehyde. Further investigation is needed in those areas as well as the effects of cinnamaldehyde concentration on sensory properties of minimally processed juices.

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Figures and Tables

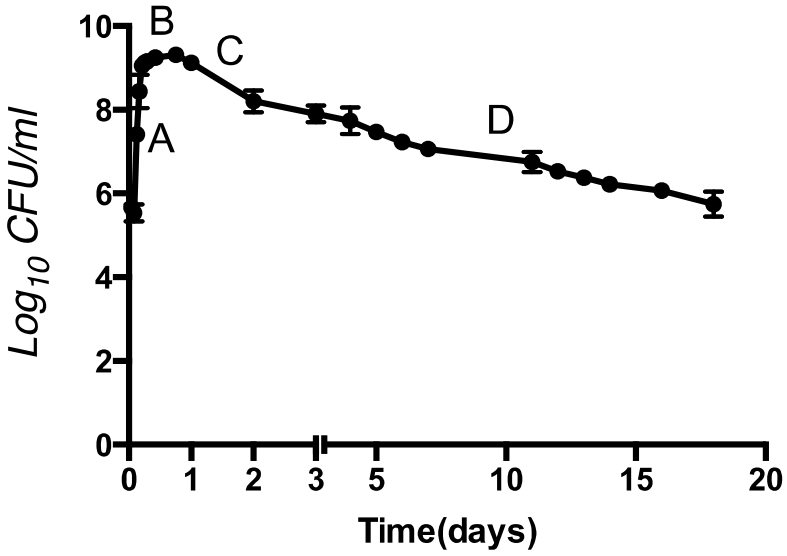

Figure 1. Growth of *Escherichia coli* O157: H7in TSBYE at 35°C for different times to yield exponential-phase (A), stationary-phase (B), death-phase (C), or LTS phase (D) cells. The data points and error bars represent means and standard deviations based on three replications of the experiment

Table 1. Antibacterial effectiveness of cinnamaldehyde against stationary-phase and LTS phase cells *E. coli* in peptone water for 72 hours

A. Viable count (log_{10} CFU/mL) of stationary phase cells of *E. coli*

Treatment (µL/mL)	2h	4h	8h	24h	48h	72h
Control	5.89±0.49[a]	5.65±0.28[a]	5.68±0.35[a]	5.52±0.31[a]	5.48±0.28[a]	5.38±0.39[a]
Cinnamaldehyde (0.25)	5.87±0.27[a]	5.68±0.44[a]	5.61±0.16[a]	5.58±0.24[a]	5.41±0.49[a]	5.35±0.18[a]
Cinnamaldehyde (0.5)	5.86±0.13[a]	5.65±0.28[a]	5.52±0.21[b]	4.78±0.34[b]	3.96±0.26[b]	2.66±0.42[b]
Cinnamaldehyde (1.0)	5.82±0.23[a]	5.51±0.32[b]	4.92±0.37[b]	3.93±0.29[b]	2.30±0.39[b]	1.90±0.31[b]
Cinnamaldehyde (1.5)	5.65±0.36[b]	5.02±0.25[b]	3.48±0.14[b]	2.14±0.36[b]	1.53±0.42[b]	ND; -ve

B. Viable count (log_{10} CFU/mL) of LTS phase cells of *E. coli*

Treatment (µL/mL)	2h	4h	8h	24h	48h	72h
Control	5.58±0.20[a]	5.61±0.24[a]	5.67±0.16[a]	5.76±0.09[a]	5.41±0.19[a]	5.09±0.18[a]
Cinnamaldehyde (0.25)	5.56±0.31[a]	5.61±0.21[a]	5.56±0.26[a]	5.38±0.31[b]	5.21±0.36[b]	5.06±0.25[a]
Cinnamaldehyde (0.5)	5.59±0.28[a]	5.49±0.19[b]	5.25±0.31[b]	4.64±0.29[b]	3.38±0.49[b]	2.06±0.39[b]
Cinnamaldehyde (1.0)	5.64±0.18[a]	4.93±0.35[b]	4.86±0.52[b]	3.15±0.15[b]	2.70±0.37[b]	1.00±0.41[b]
Cinnamaldehyde (1.5)	5.18±0.19[b]	4.81±0.47[b]	3.82±0.45[b]	2.34±0.13[b]	1.60±0.22[b]	ND; -ve

Initial viable count of *E. coli*: 6.01 ± 0.05 log10 CFU/mL.

Values are means ± standard deviations from three replications.

[b] Means with a different letter within a column differ significantly ($p < 0.05$).

CFU, colony-forming unit; ND, no colonies detected on agar plates with the lowest dilution (1:3) of the sample; -ve, negative enrichment test.

Table 2. Antibacterial effectiveness of cinnamaldehyde against stationary-phase and LTS phase cells *E. coli* in carrot juice for 72 hours

A. Viable count (log_{10} CFU/mL) of stationary phase cells of *E. coli*

Treatment (µL/mL)	2h	4h	8h	24h	48h	72h
Control	5.85±0.23 [a]	5.67±0.34 [a]	5.67±0.16 [a]	5.69±0.47 [a]	5.68±0.25 [a]	5.67±0.30 [a]
Cinnamaldehyde (0.25)	5.82±0.31 [a]	5.66±0.25 [a]	5.63±0.41 [a]	5.55±0.29 [b]	5.56±0.37 [b]	5.54±0.26 [b]
Cinnamaldehyde (0.5)	5.86±0.11 [a]	5.65±0.29 [a]	5.58±0.31 [b]	5.48±0.25 [b]	5.08±0.16 [b]	4.56±0.37 [b]
Cinnamaldehyde (1.0)	5.87±0.22 [a]	5.58±0.36 [b]	5.51±0.21 [b]	5.38±0.13 [b]	4.45±0.35 [b]	3.19±0.44 [b]
Cinnamaldehyde (1.5)	5.72±0.21 [b]	5.10±0.28 [b]	5.08±0.26 [b]	4.78±0.35 [b]	3.89±0.39 [b]	2.35±0.31 [b]

B. Viable count (log_{10} CFU/mL) of LTS phase cells of *E. coli*

Treatment (µL/mL)	2h	4h	8h	24h	48h	72h
Control	5.51±0.15 [a]	5.59±0.21 [a]	5.61±0.28 [a]	5.71±0.29 [a]	5.42±0.26 [a]	5.19±0.38 [a]
Cinnamaldehyde (0.25)	5.64±0.33 [b]	5.68±0.27 [b]	5.67±0.16 [a]	5.68±0.39 [a]	5.38±0.41 [a]	5.08±0.32 [b]
Cinnamaldehyde (0.5)	5.77±0.22 [b]	5.60±0.35 [a]	5.56±0.39 [a]	5.15±0.43 [b]	4.87±0.37 [b]	4.04±0.28 [b]
Cinnamaldehyde (1.0)	5.79±0.05 [b]	5.34±0.21 [b]	5.46±0.41 [b]	4.62 ±0.29 [b]	3.25±0.13 [b]	2.48±0.45 [b]
Cinnamaldehyde (1.5)	5.75±0.46 [b]	5.38±0.39 [b]	5.30±0.37 [b]	3.79±0.24 [b]	2.70±0.29 [b]	1.61±0.26 [b]

Initial viable count of *E. coli*: 6.01 ± 0.05 log10 CFU/mL

Values are means ± standard deviations from three replications.

[b] Means are all significantly different ($P < 0.05$).

CFU, colony-forming unit; ND, no colonies detected on agar plates with the lowest dilution (1:3) of the sample; -ve, negative enrichment test.

Table 3. Antibacterial effectiveness of cinnamaldehyde against stationary-phase and LTS phase cells *E. coli* in pH-adjusted peptone water for 48 hours

A. Viable count (log_{10} CFU/mL) of stationary phase cells of *E. coli*

Treatment (µL/mL)	1h	2h	4h	8h	24h	48h
Control	5.81±0.03 [a]	5.84 ±0.12 [a]	5.77±0.16 [a]	5.76±0.27 [a]	5.68±0.21[a]	5.55±0.21 [a]
Cinnamaldehyde (0.25)	5.81±0.05 [a]	5.60±0.22 [b]	5.58±0.37 [b]	5.38±0.21 [b]	5.15±0.31 [b]	5.08±0.18 [b]
Cinnamaldehyde (0.5)	5.79±0.11 [a]	5.53±0.29 [b]	5.46±0.25 [b]	5.23±0.37 [b]	5.00±0.29 [b]	4.38±0.25 [b]
Cinnamaldehyde (1.0)	5.82±0.09 [a]	5.41±0.35 [b]	4.95±0.36 [b]	3.20±0.11 [b]	2.69±0.26 [b]	2.29±0.16 [b]
Cinnamaldehyde (1.5)	5.81±0.07 [a]	5.24±0.27 [b]	4.80±0.21 [b]	2.80±0.28 [b]	2.15±0.19 [b]	ND; -ve

B. Viable count (log_{10} CFU/mL) of LTS phase cells of *E. coli*

Treatment (µL/mL)	1h	2h	4h	8h	24h	48h
Control	5.73±0.05 [a]	5.72 ±0.15 [a]	5.73±0.11[a]	5.62±0.29 [a]	5.50±0.37 [a]	5.46±0.26[a]
Cinnamaldehyde (0.25)	5.74±0.11 [a]	5.74±0.23 [a]	5.65±0.29 [a]	5.32±0.41 [b]	5.18±0.21 [b]	4.44±0.15 [b]
Cinnamaldehyde (0.5)	5.65±0.12 [a]	5.63±0.12 [b]	4.74±0.09 [b]	4.31±0.28 [b]	3.56±0.31 [b]	1.77±0.34 [b]
Cinnamaldehyde (1.0)	4.87±0.08 [b]	3.11±0.16 [b]	2.82±0.28 [b]	1.34±0.31 [b]	ND; -ve	ND; -ve
Cinnamaldehyde (1.5)	4.81±0.05 [b]	2.88±0.22 [b]	1.70±0.18 [b]	ND; -ve	ND; -ve	ND; -ve

Initial viable count of *E. coli*: 6.01 ± 0.05 log10 CFU/mL.

Values are means ± standard deviations from three replications.

[b] Means are all significantly different ($P < 0.05$).

CFU, colony-forming unit; ND, no colonies detected on agar plates with the lowest dilution (1:3) of the sample; -ve, negative enrichment test.

Table 4. Antibacterial effectiveness of cinnamaldehyde against stationary-phase and LTS phase cells *E. coli* in apple juice for 48h

A. Viable count (log_{10} CFU/mL) of stationary phase cells of *E. coli*

Treatment (µL/mL)	1h	2h	4h	8h	24h	48h
Control	5.73±0.34 [a]	5.67±0.07 [a]	5.71±0.19 [a]	5.69±0.36 [a]	5.68±0.05 [a]	5.67±0.03 [a]
Cinnamaldehyde (0.25)	5.38±0.13 [b]	5.26 ±0.28 [b]	5.25±0.67 [b]	5.06±0.22 [b]	4.63±0.44 [b]	3.59±0.15 [b]
Cinnamaldehyde (0.5)	5.06±0.66 [b]	4.78±0.34 [b]	4.15±0.29 [b]	3.56±0.46 [b]	2.54±0.21 [b]	1.25±0.29 [b]
Cinnamaldehyde (1.0)	4.85±0.45 [b]	3.35±0.86 [b]	2.62±0.58 [b]	1.00±0.39 [b]	ND; -ve	ND; -ve
Cinnamaldehyde (1.5)	4.45±0.71 [b]	3.01±0.36 [b]	2.25±0.76 [b]	ND; -ve	ND; -ve	ND; -ve

B. Viable count (log_{10} CFU/mL) of LTS phase cells of *E. coli*

Treatment (µL/mL)	1h	2h	4h	8h	24h	48h
Control	5.78±0.21 [a]	5.81±0.15 [a]	5.76±0.13 [a]	5.54±0.24 [a]	5.52±0.28 [a]	5.50±0.07 [a]
Cinnamaldehyde (0.25)	5.81±0.27 [a]	5.79 ±0.32 [a]	5.69±0.21 [a]	5.51±0.42 [a]	5.50±0.15 [a]	4.87±0.46 [b]
Cinnamaldehyde (0.5)	5.56±0.13 [b]	5.08±0.39 [b]	4.81±0.11 [b]	4.55±0.40 [b]	3.96±0.12 [b]	2.78±0.54 [b]
Cinnamaldehyde (1.0)	5.57±0.07 [b]	4.97±0.15 [b]	4.52±0.46 [b]	3.88±0.39 [b]	2.63±0.56 [b]	1.81±0.42 [b]
Cinnamaldehyde (1.5)	5.22±0.25 [b]	4.82±0.47 [b]	3.22±0.35 [b]	2.26±0.12 [b]	1.70±0.41 [b]	ND; -ve

Initial viable count of *E. coli*: 6.01 ± 0.05 log10 CFU/mL.

Values are means ± standard deviations from three replications.

[b] Means are all significantly different ($P < 0.05$).

CFU, colony-forming unit; ND, no colonies detected on agar plates with the lowest dilution (1:3) of the sample; -ve, negative enrichment test.

CHAPTER 6 ANTIBIOTIC RESISTANCE, PROTEIN SYNTHESIS AND MORPHOLOGICAL CHANGES IN LONG-TERM-SURVIVAL CELLS OF *SALMONELLA* TYPHIMURIUM ATCC 14028

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Fei Wang[2], Aubrey Mendonça[1,2]*, Byron Brehm-Stecher[1,2], Alan DiSpirito[3], and Emalie Thomas-Popo[1,2]

[1]Department of Food Science and Human Nutrition, and [2]Interdepartmental Microbiology Graduate Program, [3]Department of Biochemistry, Biophysics, and Molecular Biology Iowa State University, Ames, Iowa 50011

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food

Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-2950. Fax: (515) 294-8181. Email: amendon@iastate.edu.

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Abstract

Resistance to antibiotics, protein synthesis and morphological changes in long-term-survival (LTS) phase cells of *Salmonella* Typhimurium ATCC 14028 were investigated. *S.* Typhimurium was grown to obtain exponential-, stationary- and long-term-survival (LTS) cells at 35 °C in tryptic soy broth with yeast extract (TSBYE). Cells were treated with ampicillin at 0.2, 0.5, 1.0, 2.0 and 3.0 mg/mL for 3 hours. The LTS phase cells were the most resistant to ampicillin with only 1.19 $\log_{10}$ CFU/mL reduction after treatment with 2.0 mg/mL ampicillin. Exponential phase and stationary phase cells were reduced by more than 5 and 3.8 $\log_{10}$ CFU/mL, respectively. The viability of LTS cells was decreased by chloramphenicol (100 μg/mL) with significant reduction in cell viability observed after 5 days of treatment. The size of cells decreased as they transitioned from the stationary to the LTS phase and rod-shaped cells turned to cocci as they entered the LTS phase. Cells in LTS phase were isolated by centrifugation, suspended in fresh TSBYE, and incubated at 35 °C. Cells transitioned from the LTS phase to stationary phase, and their shapes changed back to from cocci to rods as well. The same LTS phase was also found in *E. coli* K12 and *Listeria monocytogenes* Scott A. We speculate that LTS cells of *S.* Typhimurium are tolerant of antibiotics, change from rods to cocci as they transition to the LTS phase, and require protein synthesis to maintain viability during LTS. Further research is needed to validate this speculation and elucidate the practical significance of these findings.

Introduction

Conditions in the natural environment seldom allow constant sustained bacterial growth and differ vastly from conditions created in the laboratory for culturing microorganisms. Laboratory conditions including nutritious growth media, optimal

incubation temperatures and pH, and growth of organisms in pure cultures are unrealistic in nature. In reality bacteria may intermittently face harsh environmental factors including build-up of metabolic waste, microbial competition, antibiotics, and nutrient depletion.

As a human enteric pathogen that cycles from the intestinal tract to the environment *Salmonella* encounters extreme and diverse environments, and therefore has developed responses to resist these adverse conditions[10]. To protect against harsh conditions, bacteria can enter a stationary phase during which their stress protection systems become activated. Also, survival of bacteria under those conditions would require that they make crucial reorganizations at the cellular and molecular levels.

Previous studies have demonstrated that bacteria did not die completely in death phase. Alternatively, a small portion of the population may enter a dormant state and display long-term survival behaviors [11]. Long-term-survival (LTS) phase has been found in *Listeria monocytogenes* [12], *Escherichia coli* [13], *Micrococcus luteus* [14] and another microorganisms [11], in which viable cell concentrations are maintained. An increasing number of pathogens such as *L. monocytogenes* persist in food processing plants for a long period of time and are resistant to environmental stresses. This poses a significant threat to both public health and the food safety because stress-hardened pathogens exhibit increased survival potential thus increasing the risk of diseases [15, 16]. Entry into a LTS phase may account for *L. monocytogenes* long-term persistence in food processing plants [12]. In the LTS phase, *L. monocytogenes* cells become coccoid-shaped, more resistant to heat and high pressure, and maintain a stable population at ~10^8 CFU/ml for at least 1 month [12].

The bacterial stationary phase is followed by death phase in laboratory broth systems, in response to environmental changes like accumulation of toxic metabolic wastes and the depletion of nutrients [13]. Bacteria in the environment enter a starved state and survive for long time until relief from starvation [11]. Starvation responses have been reported including formation of periplasmic spaces [17], shrinkage in cells size [18], protein degradation [19], reduction in respiration rate [20], reduction in RNA levels [21], stable or increased DNA level [22], reduction in microbial diversity in habitat [23], changes in levels of carbohydrates [24], production of specific antigens [25], decrease in adenylate energy charge [26], decrease in ATP [27] and changes in fatty acid profiles [28]. During long-term-survival foodborne pathogens may likely exhibit characteristics typical of those observed in persister cells as described in the medical area.

Persister cells are bacterial cells that exhibit resistance to antibiotics which prevent the synthesis of cell wall (peptidoglycan) or DNA. They survive antibiotic treatments owing to their dormant nature, but they "awaken" and start growing upon the removal of antibiotics, which explains the fact that they are responsible for various chronic and recurrent infections [29-31]. They however remain sensitive to antibiotics when they are growing. Persister cells enter a dormant state involving a global slowdown of metabolic processes [29]. To date, persister cell research has been conducted with cells in the exponential-to-stationary phases [29, 32, 33], but not with cells in the LTS phase where an almost whole population (not a sub-population) of persisters can be generated. Knowledge of cellular processes in the LTS phase is important for both fundamental and practical perspectives. For the human enteric pathogens such as *Samonella* in the LTS phase may be induce dormancy, pathogen resistance to antibiotics, sustenance of protein

synthesis to maintain viability, and morphological changes. Therefore, the objective of present study is to investigate antibiotic resistance, protein synthesis and morphological changes in long-term-survival (LTS) phase cells of *Salmonella* Typhimurium ATCC 14028.

Materials and Methods

Bacterial cultures and culture conditions

Salmonella enterica serovar Typhimurium ATCC 14028, *E. coli* K12 and *Listeria monocytogenes* Scott A were used in the study and obtained from the culture collection of the Microbial Food Safety Laboratory of Iowa State University. Each glycerol stock was maintained at -80 °C and was transferred at least twice in 10 mL of tryptic soy broth with 0.6% yeast extract (TSBYE; Difco Laboratories, Detroit, MI), with incubation at 35 °C for 24 hours prior to each experiment.

Preparation of exponential-, stationary- and long-term survival cells

Portions (1.0 mL) of *S.* Typhimurium, *E. coli* K12 and *L. monocytogenes* culture were transferred to TSBYE (100 mL) in screw-capped 250 mL Erlenmeyer flasks. The inoculated medium was incubated at 35 °C under shaking at 150 rpm in a gyrorotary shaker incubator (New Brunswick Scientific Co. Inc., Edison, NJ) for 3 and 18 hours to obtain exponential- and stationary-phase cells, respectively. The bacteria were cultured in TSBYE (100 mL) in screw-capped 250 mL Erlenmeyer flask and incubated statically at 35 °C in incubator for 15-30 days to obtain LTS cells. Cultures (30 mL each) were harvested by centrifugation (10,000 × g, 10 min, 4 °C) using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT), and washed once in 0.85% (w/v) saline. Pelleted cells were suspended in fresh saline to achieve a final viable cell concentration of ~7.0 $\log_{10}$ CFU/mL. Colony counts of the washed cell suspensions

were evaluated by serially diluting (10-fold) and surface plating samples on tryptic soy agar (Difco; Becton Dickinson) that was supplemented with 0.6% yeast extract (TSAYE) followed by counting bacterial colonies on TSAYE after incubation (35 °C) for 24 hours.

Exposure to ampicillin treatment

A commercial powder ampicillin was purchased from Sigma-Aldrich, St. Louis, MO. Treatment solutions of ampicillin were prepared in 10 mL of 0.85% (w/v) NaCl (saline) to get 0.2, 0.5, 1.0, 2.0 and 3.0 mg/mL. Each treatment solution was sterilely filtered using disposable Nalgene Type S Sterilization Filter Units (0.22 µm pore size) (Curtin Matheson Scientific, Inc, Broadview Heights, OH), aseptically transferred into separate, sterile bottles and held at ambient temperature (23 ± 1 °C) prior to inoculation. Saline without addition of ampicillin served as control. The control and each solution of ampicillin were inoculated with 1.0 mL of cell suspension to give a final concentration of ~ 5.88 Log_{10} CFU/ mL. Cells were mixed immediately by vortexing and then held at ambient temperature. Aliquots (1.0 or 0.1 mL) of treated cell suspensions were removed from the treatment tubes after 3 hours and washed by centrifugation (10,000 × g, 10 min, 4 °C) in 1 mL saline. Then they were added into 9 mL buffered peptone water (BPW, Difco), mixed by vortexing, and then surface plated (in duplicate) onto TSAYE. Agar plates were incubated at 35 °C, and bacterial colonies were counted at 24 hours.

Inhibition experiment and viability determination

Chloramphenicol powder was bought from Sigma-Aldrich, St. Louis, MO and prepared in 100 mL of 0.85% (w/v) NaCl (saline) to obtain a concentration of 100 µg/mL. The chloramphenicol solution was sterilely filtered using disposable Nalgene Type S Sterilization Filter Units (0.22 µm pore size), aseptically transferred into separate, sterile bottles and held at ambient temperature (23 ± 1 °C) prior to inoculation. Treatment

solutions were saline with added chloramphenicol (100 µg/mL); saline solution with no chloramphenicol served as control. The control and treatment solutions were inoculated with 1.0 mL of cell suspension to give a final concentration of ~ 6.60 Log_{10} CFU/ mL. Cells were mixed immediately by vortexing and then held at 22 ± 1 °C. The viability of LTS cell suspensions was determined after the addition of chloramphenicol at day 0, 2, 4, 6, 8, 10, 12, 14 and 16. Aliquots (1 or 0.1 mL) of treated cell suspensions were removed from control and treatment tubes at each time point and added into 9 mL BPW, mixed by vortexing, and then surface plated (in duplicate) onto TSAYE. Agar plates were incubated at 35 °C, and bacterial colonies were counted at 24 hours.

Changes of viable cell density for 810 days

Listeria monocytogenes and E. coli K12 cell suspensions were prepared for the determination of cell viability. Serial dilutions of the cell suspension were prepared in BPW at appropriate intervals during 810 days in TSBYE, and 0.1 mL aliquot of appropriate dilutions were surface plated on TSAYE. All inoculated agar plates were incubated aerobically at 35 °C and the bacterial colonies were counted at 24 hours.

Light microscopy of Gram stain and transmission electron microscopy

Transmission electron microscopy was used to view *Salmonella* Typhimurium cells at different ages including stationary-phase cells (18 hours), LTS phase cells at 1 month, 22 months and 42 months, as well as 42-month old LTS cells in fresh TSBYE, for their morphology changes. All cells were harvested in 0.85% (w/v) saline at 23 ± 1 °C by centrifugation at 10,000 × g, 10 min, 4 °C. Gram stains of cell cultures were examined with a × 100 oil immersion objective lens using a Leica DM100 light microscope (Leica, Buffalo Grove, United States). Aliquots (3 µL each) of cells in saline were placed onto a carbon film coated copper grid for 1 minute. The supernatant was removed by wicking

from the side with a piece of filter paper and 3 µL of 2% aqueous uranyl acetate was placed onto the grid for 30 seconds. The stain was removed by wicking and the grid was allowed to dry. Images were made using a JEOL 2100 scanning transmission electron microscope at an accelerating voltage of 200 kV (Japan Electron Optics Laboratory, USA, Peanody, MA)

Transmission from LTS phase back to stationary phase

The protocol has been reported by Wen et, al [12]. *S.* Typhimurium was incubated in TSBYE at 35 °C for 42 months and then 10 mL of the culture was centrifuged at 10,000× g for 10 min at room temperature (~ 23 °C). Pellets were suspended in 10 mL of fresh TSBYE to get a starting concentration of ~10^8 CFU/mL and then incubated at 35 °C for 9 hours to obtain cells in stationary phase.

Statistical analysis

Three replications of each experiment were performed. Mean numbers of viable survivors were statistically analyzed using SAS statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Significant differences were defined at $P < 0.05$ for all the experimental data.

Results and Discussion

Ampicillin resistance of exponential-, stationary- and LTS phase cells of *S.* Typhimurium is shown in Figure 1. The log reduction of exponential-phase cells of *S.* Typhimurium was 3.39 $\log_{10}$ CFU/mL with the concentration of ampicillin at 0.2 mg/mL. In contrast, the log reduction of stationary- phase and LTS phase cells were 0.41 and 0.55 $\log_{10}$ CFU/mL, respectively, rendering no significant difference with each other (P <0.05). At an ampicillin concentration of 2.0 mg/mL, the entire exponential-phase cells were completely killed. The log reduction of stationary phase cells was 3.8 $\log_{10}$

CFU/mL whereas LTS phase cells showed only 1.19 $\log_{10}$ CFU/mL reduction. These results clearly indicate that LTS phase cells are the most resistant cells over exponential-phase and stationary-phase cells of *S.* Typhimurium against ampicillin.

Survivors of LTS phase cells of *S.* Typhimurium with and without chloramphenicol treatment are shown in Figure 2. At day 0, 6.60 $\log_{10}$ CFU/mL LTS phase cells were treated with chloramphenicol (treatment) or suspend in saline without chloramphenicol (control). There was no significant difference between control and treatment in the first 5 days. However, more reduction for treatment cells began after 5 days. At day 10, the survivors of LTS phase cells in control were still ~ 6.60 $\log_{10}$ CFU/mL, while only 4.79 $\log_{10}$ CFU/mL LTS phase cells survived upon treatment. At day 16, all LTS phase cells were killed by chloramphenicol while 6.29 $\log_{10}$ CFU/mL LTS cells still survived in control. These results indicate that LTS phase cells of *S.* Typhimurium are inactivated by chloramphenicol an inhibitor of protein synthesis. These results therefore suggest that protein synthesis is important for the sustained viability of LTS *S.* Typhimurium cells.

The data presented herein show that LTS phase cells of *S.* Typhimurium are not completely "dormant" but maintain a low level of metabolic activity like protein synthesis. In LTS phase, "young" cells are constantly replacing "old" cells as the total number of cells remain constant over time [13]. Lewis et, al. also found that persister cells are not dormant without cell-wall synthesis, translation or topoisomerases activity since antibiotics still can corrupt and take function on their target molecules for persister cells [29]. Persister cells of *E. coli* still possesses low level of translation, which enables differential cell sorting base on the expression of a detectable protein [29]. In this study,

protein synthesis inhibitor, chloramphenicol did not exert significant effects on the survival of LTS cells in the beginning of test and there was no difference between the cells with or without treatment of chloramphenicol. However, LTS cells treated by chloramphenicol showed a dramatic reduction after treatment for 5 days. We speculated that LTS cells still have a low of protein synthesis activity. In the beginning of inhibition, only few proteins might be required to keep survival or protect the core DNA of cells. With the increasing time of inhibition, required proteins are used up and cannot be reproduced due to chloramphenicol. The treated LTS cells cannot remain viable without limited protein synthesis and showed reduction in viable population. Further investigation on which proteins or amino acids are required for the survival of LTS cells is currently ongoing in our laboratory.

Representative photomicrographs of cells of *S.* Typhimurium in different phases are shown in Figure 3. Gram stains and TEM analysis showed that cells decreased in size as they transitioned from stationary phase to LTS phase (Fig. 3, LM and TEM). TEM images indicated that cells in stationary phase were rod-shaped, while cells in LTS phase at 1, 22 and 42 months were all cocci-shaped. TEM images revealed cells in LTS phase at 1, 22 and 42 months with a dark thin cell wall and a network in the cytoplasm, which were condensed and attached to the cell wall (Fig. 3, TEM). Cells in LTS phase at 42 months showed even more condensed cytoplasm than cells at 1 and 22 months. When cells of *S.* Typhimurium in LTS phase after 42 months of incubation were inoculated into fresh, sterile TSBYE and incubated at 35 °C, cells transited to stationary phase (Fig. 3E). The shape of cells changed from cocci to rod (Fig. 3E LM and TEM) and cytoplasm also went back to uniform status in stationary phase (Fig. 3E TEM).

It has been shown that starvation can induce a change in cell shape from rods to cocci in *Arthrobacter crystallopoietes* [37], *A. globiformis* [38], a marine vibrio [39], and *Rhizobium leguminosarum* [40]. Starvation also induced the formation of smaller coccoid cells of *Staphylococcus aureus* [41]. Previous studies shown that after glucose starvation, cells of *L. monocytogenes* were shorter and wider than log-phase cells [42]. The formation of cocci may be due to cell division without an increase in total biomass [40, 43, 44]. Cocci could be formed by cell shrinkage and cytoplasmic condensation [43], which might have caused the textured surface of cocci seen in the present study (Fig. 3, TEM). Cytoplasmic condensation might lead to lowered water activity by decreasing cell volume and water content, and/or by increasing solute concentration. Smaller cocci have a larger surface-to-volume ratio than rods, which can enhance simple or facilitate diffusion for nutrient uptake, reducing the need for energy in nutrient transportation during starvation [45]. After being inoculated into fresh TSBYE, the shape of cells changed back to rod from cocci. Barotolerance and thermotolerance of *L. monocytogenes* cells in the LTS phase significantly decreased when they transferred into fresh broth and re-entered the log phase from LTS phase [45]. They also found that there was weak correlation between cell length and resistance when cells transitioned from rods to cocci, possibly because resistance was due to both synthesis of stress proteins and cytoplasmic condensation. By contrast, a strong correlation was observed when cocci transitioned to rods, which may due to the loss of resistance after rehydration of the cytoplasm without synthesis of stress proteins. Similar observations have been also reported in *Bacillus cereus* spores [46], *L. monocytogenes* [12], A. crystallopoietes [37] and marine

microorganisms [47]. The rod-coccus-rod life cycle of *S.* Typhimurium is similar to that of other microorganisms that inhabit natural environments.

Growth curves of *Listeria monocytogenes* and *E. coli* K12 incubated in TSBYE for 810 days at 35 °C were depicted in Figure 4A and 4B. The same with *S.* Typhimurium, cells for both microorganisms went through log phase in 1 day. The highest population reached 8.37 and 9.39 $\log_{10}$ CFU/mL for *L. monocytogenes* and *E. coli* K12, respectively. Their viable counts remained stable for ca.2 days, which was stationary phase and began to die after 2 days and entered death phase. The viable population dropped rapidly to 7.6 and 8.5 $\log_{10}$ CFU/mL for *L. monocytogenes* and *E. coli* K12, respectively, which resulted in ~90% loss of cell viability. The death of cells ceased and the viable counts were relatively stable at 5- 6 $\log_{10}$ CFU/mL up to 810 days without any supplying additional of nutrients, and this period was the long-time-survival (LTS) phase.

The findings of this research are that LTS S. Typhimurium is resistant to ampicillin, requires protein synthesis to maintain viability, and transforms from rod to coccoid shape as it transitions into the LTS phase. Also the pathogen can revert back to rod shaped cell if placed into fresh growth media. Those three findings on LTS *S.* Typhimurium certainly represent only a few of the many characteristics of that pathogen in a non-growing state. Perhaps the lack of cell multiplication is a contributory factor to the observed antibiotic resistance. This is supported by the fact that ampicillin, the antibiotic used in this study, blocks cell wall (peptidoglycan) synthesis; therefore, its effect would certainly be negated if the cells are not growing and actively synthesizing cell wall.

With regard to loss of cell viability in the presence of chloramphenicol two issues need to be considered in terms of the LTS phase. The first is that in the LTS phase bacteria might be dormant but maintaining a low level of protein synthesis to keep alive. Because *S*. Typhimurium cannot form spores which are metabolically inactive in the resting (non-germinating) state it has to sustain some metabolic activity to remain alive. The LTS phase is not an optimum condition for bacteria; therefore, some amounts of stress are imposed on the cells during LTS. Under stress bacterial antitoxins which are important in neutralizing toxins that can cause cell death, are readily degraded. In this respect it is important for the LTS bacterial cell to replenish degraded antitoxin, a part of the toxin-antitoxin system, or the cell will die. Therefore, protein synthesis becomes crucial for the making adequate amounts of the labile antitoxin to keep the cell alive. Another issue is that the LTS phase is possibly a metabolically dynamic phase with cell populations changing over time [13]. In this situation newly formed mutant cells which are more competitive that the parent cells eventually take over the whole cell population. Protein synthesis will also be required to maintain the required metabolism for these changes in cell populations.

Recent studies in our laboratory point to a strong association of the coccoid form of S. Typhimurium with pathogen resistance to several antimicrobial processes including UV radiation, desiccation, and heating during hot air drying. Wen et al [12] demonstrated enhanced resistance of *L. monocytogenes* to high pressure and heating as that pathogen transitioned from exponential to LTS phase with the cells changing to coccoid forms. We speculate that the compact coccoid cell shape with apparently

condensed cytoplasm is a fitness strategy of non-spore-forming bacteria that permits long term survival in nature.

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Figures and Tables

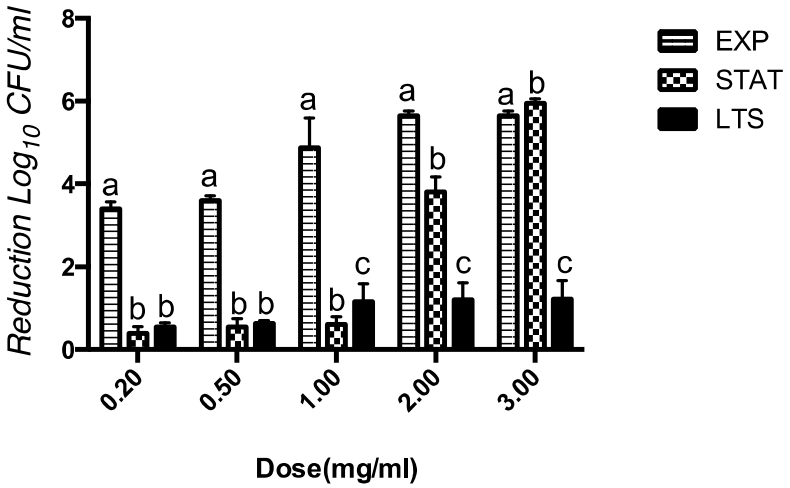


Figure 1. Reduction of three physiological states (exponential, stationary and LTS) of *S.* Typhimurium working against ampicillin. Populations are the means of three values ± SDs. Error bars represent SDs (n = 3, i.e., three independent experiments). For each exposure time point, bars that do not share a common letter are significantly different ($p < 0.05$).

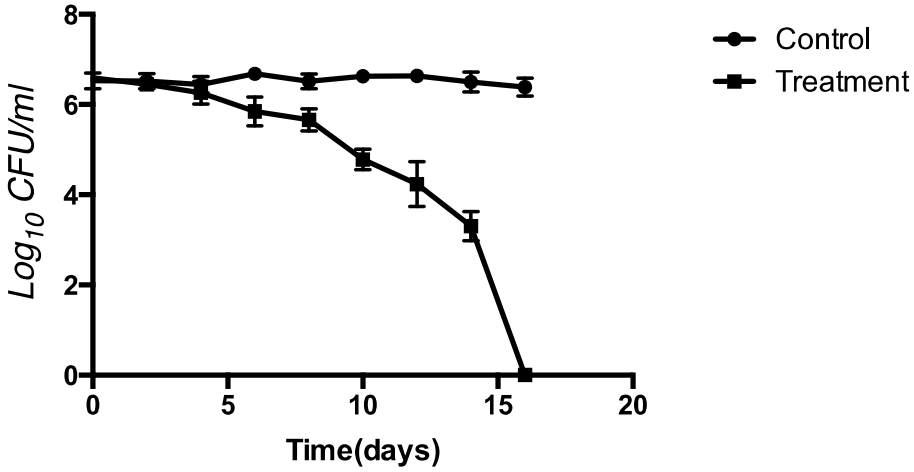


Figure 2. Survival of LTS phase of *S.* Typhimurium treated with 100 µg/ml chloramphenicol (treatment) or no chloramphenicol (control) in 0.85% saline at 25 °C. Populations are the means of three values ± SDs. Error bars represent SDs (n = 3, i.e., three independent experiments)

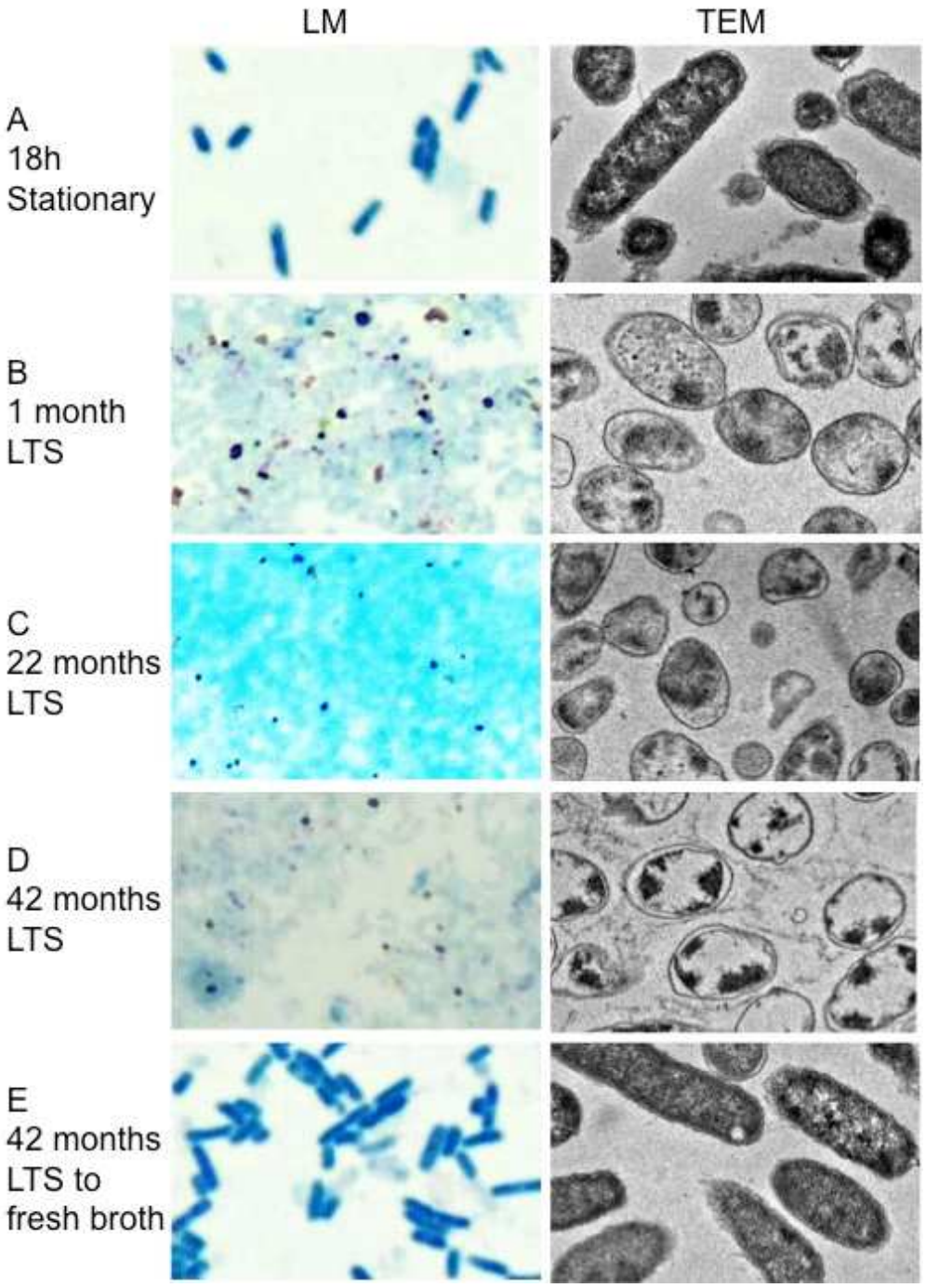
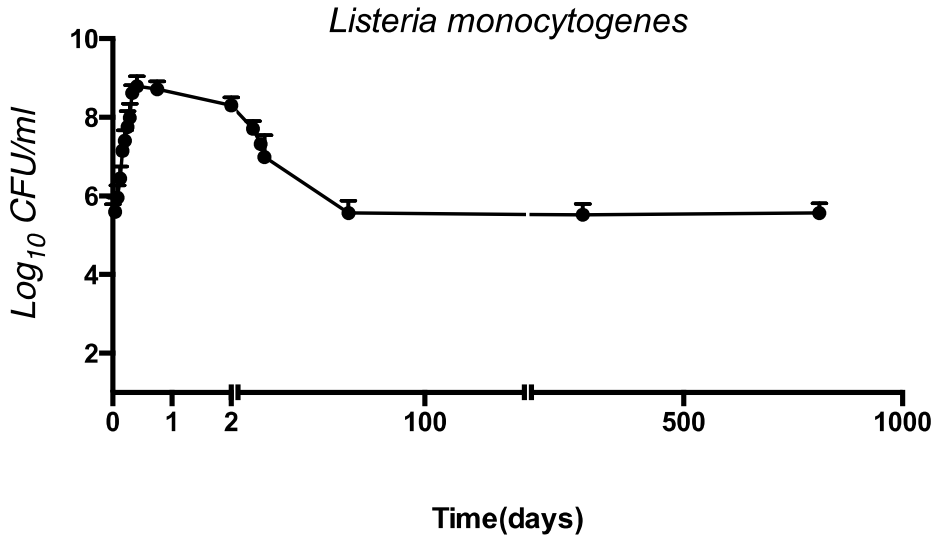


Figure 3. LM and TEM images of *S.* Typhimurium at different growth phases. Cells of *S.* Typhimurium ATCC 14028 were grown in TSBYE at 35 °C for various times to yield cells at different phases and then observed using LM (Gram stain) and TEM. Bars: 10 μm (LM) and 1 μm (TEM)

A



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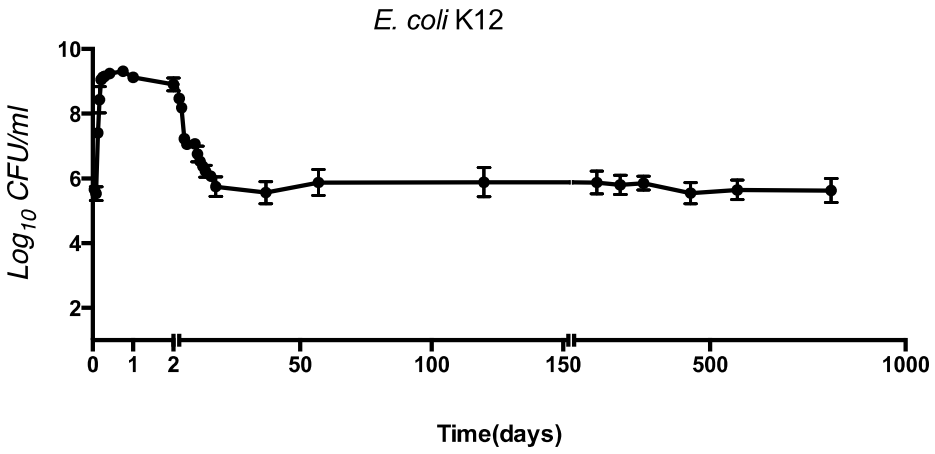

Figure 4. Growth of *Listeria monocytogenes* Scott A (A) and *E. coli* K12 (B) in TSBYE at 35°C for different times to yield exponential-phase, stationary-phase, death-phase and or LTS phase. The data points and error bars represent means and standard deviations based on three replications of the experiment.

CHAPTER 7. GENERAL CONCLUSION

The present studies revealed that *Salmonella* Typhimurium, *Salmonella* PT 30, *E. coli* O157: H7, *E. coli* K12 and *Listeria monocytogenes* can form cocci in long-term-survival state. The LTS cells of *S.* Typhimurium were significantly more resistant to UV radiation in 0.85% saline and apple juice. Both *S.* Typhimurium and *S.* PT 30 in the LTS phase are highly resistant to desiccation on paper discs and raw almonds at ambient temperature, and also to hot air drying on almonds at 100°C. The *E. coli* O157: H7 LTS cells were the most resistant to cinnamaldehyde, a natural antimicrobial of plant origin. Cells of *S.* Typhimurium in LTS state transferred from rod shape to cocci shape and behaved like persister cells, including antimicrobial resistant to ampicillin and low metabolism activity.

Additional serotypes of *Salmonella, E. coli*, *Listeria* and other common foodborne pathogens should be evaluated in the future to confirm that morphological change from rods to cocci and resistance to UV radiation, desiccation and natural antimicrobials in the LTS phase of the bacterial life cycle are general phenomenon in other microorganisms. Microbial food safety studies on the resistance of pathogenic bacteria antimicrobial interventions are usually based on the use of stationary-phase cells, which might not be the most resistant. This statement is substantiated by research results presented on LTS pathogens used in the present studies. Therefore, the food processing and preservation methods designed on the basis of inactivating stationary phase can be unreliable in ensuring food safety as they are likely to overestimate the extent of bacterial inactivation. Furthermore, LTS cells of pathogens may persist in food processing facilities especially

in hard-to-clean sites or in biofilms and serve as a continuous source of contamination to newly processed food products.