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Previous stress exposures influence subsequent UV-C resistance of *Salmonella enterica* in coconut liquid endosperm

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ABSTRACT

This study determined the effects of prior exposures to stresses commonly encountered by cells in food production and processing environments on the subsequent UV-C inactivation rates of *Salmonella enterica* in coconut liquid endosperm. Seven different strains of *S. enterica* were separately exposed to individual stresses such as gradual acidification (final pH 4.5), abrupt desiccation (a_w 0.96), or heat stress at 40 °C for 24 h, after which the test strains were cocktailed and subjected to UV-C challenge. Cells were also exposed to all possible combinations of the individual stresses and thereafter subjected to UV-C challenge. Cells exposed to all individual and combinations of stresses exhibited 1st order, log-linear inactivation behavior (R^2 0.903 to 0.998). Cells previously exposed to singular heat stress had the highest UV-C resistance (D_{UV-C} 43.8 mJ/cm^2), while cells exposed to all simultaneous pH, a_w, and heat stresses had the least (D_{UV-C} 22.5 mJ/cm^2). Heterologous adaptive mechanisms were observed after *S. enterica* cells were exposed to acid, acid + desiccation, heat, and acid + heat, with individual heat stress exposure resulting in the significantly most UV-C resistant cells. Results obtained in this study provide baseline information on the selection of appropriate challenge organism for establishment of UV-C process schedule for coconut liquid endosperm and similar commodities.

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1. Introduction

Fruit juices have long been regarded safe from disease-causing microorganisms due to their inherent high acidity that is unfavorable for microbial survival, growth and proliferation. However, recent outbreaks of infections of pathogens such as *Escherichia coli* O157:H7, and *Salmonella enterica* due to unpasteurized fruit juice consumption have emphasized the need for food safety systems for consumer protection (Vojdani, Beuchat, & Tauxe, 2008). The United States Food and Drug Administration (USFDA) therefore ratified the Federal Juice Hazard Analysis Critical Control Point that mandates manufacturers to subject their products to processes to reduce the population of an appropriate reference organism by 5 logarithmic cycles (USDA and USDHHS, 2010).

Thermal processing is a common means employed to decontaminate fruit juices of disease- and spoilage microorganisms. This technology is considered relatively simple, affordable, and effective in rendering finished products safe and shelf stable (Buchanan & Edelson, 1999; Mak, Ingham, & Ingham, 2001). However, the application of high temperature to heat-labile raw materials such as fruit juices results in the deterioration of physicochemical, nutritional, and sensory quality attributes of the finished products. Thus, nonthermal fruit juice processing technology alternatives must be developed.

Bactericidal ultraviolet-c (UV-C) irradiation is a nonthermal processing technology that have been approved by the USFDA for fruit juice processing applications (USFDA, 2012). Exposure of cells to UV-C rays results in the physicochemical alterations in the genetic materials that lead to inactivation. However, just like any emerging food processing technology, factors affecting UV-C efficacy must be well studied. The antimicrobial efficacy of UV-C have been previously reported to be affected by intrinsic food properties such as turbidity (Shama, 1999), and the presence of soluble (Koutchma, Keller, Chirtel, & Parisi, 2004) and insoluble solids (Murakami, Jackson, Madsen, & Schickedanz, 2006) that attenuate UV-C penetration into the food system (Guerrero-Beltran &

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Barbosa-Canovas, 2004). Furthermore, extrinsic process-related parameters such as UV-C lamp energy dose and other equipment-dependent settings have also been shown to affect UV-C efficacy (Bolton & Linden, 2003; Koutchma, 2014). The effects of microorganism-related implicit characteristics on the bactericidal activity of UV-C are possibly the least studied factors. Microorganisms in the raw materials are exposed to a number of environmental stresses in the earlier stages of the food production pipeline, which could induce adaptive mechanisms towards the processing technology that supposed to inactivate them (Gabriel, 2014).

While a numerous works on the effects of cell physiological states (*e.g.* stress-adapted and non-adapted physiological states) on the thermal resistance of microorganisms in fruit juices (Buchanan & Edelson, 1999; Gabriel, 2012b; Ryu & Beuchat, 1998), there is a dearth of information on the effects of cell physiology on its resistance towards UV-C. In a recent comprehensive review of Koutchma (2014) on the application of UV as a food processing technology, there was no discussion on screening and selection of appropriate target microorganism for UV-C processing of specific food commodities. Identification of an appropriate bactericidal-resistant species, strain, or physiology is an important step in establishing novel process schedules and for the evaluation of performance of existing ones. Inactivation of the resistant target organism would similarly result in the inactivation of the less resistant microorganisms and ensure safety or shelf stability.

Coconut liquid endosperm and beverage products derived from it are commodities that could be treated with UV-C due to the raw material sensitivity towards the traditional high temperature processing conditions. Coconut liquid endosperm undergoes quality deterioration upon subjection to high temperature (Prades, Dornier, Diop, & Pain, 2012), which may prevent manufacturers from processing the commodities, making them likely vectors of foodborne illnesses, and compromising shelf stability and profit. There have been no reports of outbreaks of infection due to coconut liquid endosperm products but its physicochemical properties, composition, and the manual nature of extracting the endosperm from the drupes increase the risk of microbial contamination, survival, proliferation, eventual consumer infection.

This study thus investigated the effects of previous exposures of *Salmonella enterica* to some commonly encountered physicochemical stresses in food production and processing environments on the subsequent UV-C inactivation rates in coconut liquid endosperm. The results obtained from this study provide baseline information on the selection of appropriate reference organism for the establishment of UV-C process schedule for specific food commodities.

2. Materials and methods

2.1. Salmonella enterica strains propagation and culture maintenance

The *S. enterica* strains that were challenged in this study included the American Type Culture Collection (ATCC) serovars Typhimurium (ATCC 14028), Diarizonae (ATCC 12325 and 29934), and Abortus-Equi (ATCC 9842). Furthermore, University of the Philippines Diliman, Laboratory of Food Microbiology and Hygiene (LFMH)-maintained cultures of *S. enterica* sers. Enteritidis, Montevideo and Infantis were also tested and used as challenge organisms.

In the preparation of working cultures, cells from each refrigerated stock culture slants were obtained and activated in nutrient broth (NB, Nutrient Broth, HiMedia, Mumbai, India), and thereafter incubated at 37 °C for 24 h. A loopful of cells from each activated culture was then transferred separately into another set of NB for enrichment at 37 °C for another 24 h. A loopful of cells from the enriched culture was then streaked onto nutrient agar (NA, Nutrient Agar, HiMedia, Mumbai, India) slant and incubated at 37 °C for another 24 h prior to refrigerated storage at 4 °C. These working cultures were sub-cultured every 14 d during the conduct of this study.

2.2. Physicochemical stress exposures

In this study, cells were exposed to different individual and combined stresses by propagating each of the test strains at sub-optimal growth conditions. The physicochemical stresses tested in this study included (1) acid stress by gradual acidification, (2) desiccation stress by a_w reduction, (3) heat stress, (4) acid + desiccation stress, (5) acid + heat stress, (6) desiccation + heat stress, and (7) acid + desiccation + heat stress. These stresses were applied to the test organisms prior to inoculation to the coconut liquid endosperm to simulate stress exposure in food production environments such as the soil, fermenting substrates, and other environments prior to being introduced to food systems such as coconut liquid endosperm, and eventual kill step during processing. Prior to stress exposures, cells from the working culture slants were subjected to the previously described activation and enrichment processes. Each of the 7 *S. enterica* serovars were individually subjected to the test stresses prior to UV-C challenge studies.

Individual stress exposures. For the gradual acid stress exposure, each of the enriched *S. enterica* serovar was loop inoculated to nutrient broth supplemented with 1% (w/v) glucose (NBG) and incubated at 37 °C for 24 h. During incubation, the cells assimilated the sugar in the growth medium and gradually liberated organic acids and lower the pH to as low as 4.46 (a_w 0.99) by the end of the incubation period (Gabriel & Nakano, 2011). For the abrupt desiccation stress, each of the enriched *S. enterica* serovars were loop inoculated into NB supplemented with NaCl (until 7% w/v) to a final a_w value of 0.96 (pH 7.2). The culture was incubated for 24 h at 37 °C. On the other hand, heat stress exposure was conducted by inoculating a loop of individual strain into separate NB (pH 7.0, a_w 0.99), which was subsequently incubated at 40 °C for 24 h.

Simultaneous stress exposures. This study also tested various combinations of stresses. For cells exposed to acid + desiccation stress, cells from each of the enriched cultures were separately transferred into NBG supplemented with NaCl and were incubated at 37 °C for 24 h. Cells that will be subjected to acid + heat stress, cells were inoculated in NBG and incubated at 40 °C for 24 h. Cells that were subjected to desiccation + heat stress were inoculated into NB with NaCl and incubated at 40 °C for 24 h. Finally, cells that were subjected to all three simultaneous stresses were inoculated in NBG with NaCl, and incubated at 40 °C for 24 h prior to subsequent UV-C inactivation studies.

2.3. Sublethal injury determination

The collective sublethal injury of all test serovars was then determined after 24 h of stress exposure. Briefly, 1 mL aliquots were obtained from each of the similarly stressed serotypes and combined in a sterile tube. The combined cells were vortexed for 10 s, and subjected to serial 10-fold dilution. To determine sublethal injury rates per stressing medium, cells were subjected to parallel plating onto NA and bismuth sulfite agar plates (BSA, Bismuth Sulfite Agar, HiMedia, Mumbai, India), which were then incubated at 37 °C for 24–48 h. Sublethal injury rate was calculated as the difference in the populations enumerated in the non-selective NA that shall allow injury repair and eventual colony formation of

injured and uninjured cells; and the selective BSA, which only allows colony formation of uninjured cells. Sublethally injured cells were defined as those that were able to multiply and form colonies on NA but not on selective media due to the damages on cell membrane and modifications in their permeability (Besse, Dubois-Brissonnet, Lafarge, & Leclerc, 2000; Hartsell, 1951; Jasson, Uyttendaele, Rajkovic, & Debevere, 2007). Equation (1) was used to calculate for the percentage of sublethal injury (SLI).

$$\% \text{ SLI} = \frac{\text{Counts on NA} - \text{Counts on BSA}}{\text{Counts on NA}} \times 100 \quad (1)$$

2.4. Suspending medium, composite inoculum preparation, and inoculation

For the UV-C inactivation studies, a commercially available coconut water (Nyogi Pure Coconut Water, Manila, Philippines) was used as the coconut liquid endosperm suspending medium in the study. Physicochemical properties were determined by using a calibrated pH meter (Eutech pH 700, Singapore, Singapore) and a digital refractometer (Milwaukee MA871, Milwaukee Instruments, Inc., North Carolina, USA) for SS. Titratable acidity (%TA, malic acid) was also determined potentiometrically using standardized 0.05 N NaOH until phenolphthalein endpoint at pH 8.1. Initial microbiological tests conducted on the suspending medium revealed that total aerobic plate and yeasts and molds counts were below detection limit (<1 CFU/mL).

After stress exposures, a cocktail of the 7 test *S. enterica* strains was prepared and used in the subsequent UV-C inactivation studies. An aliquot of 2 mL was obtained from each of the strains previously exposed to physicochemical stresses, combined into a sterile test tube, and thereafter subjected to vortex-mixing for 30 s. An aliquot of 1 mL was obtained from the composited *S. enterica* cells and transferred into a sterile microcentrifuge tube. Cells were harvested by spinning at 2419 G for 15 min using a portable centrifuge (Mini Centrifuge 17307-00, Cole Parmer, Illinois, USA). The supernatant was then decanted and cell pellet resuspension was done by vortex-mixing in 1 mL coconut liquid endosperm for 30 s. Cells were acclimatized in the suspending medium for no longer than 15 min. Only cells subjected to these previously described activation, enrichment and acclimatization steps were used in subsequent UV-C challenge studies.

2.5. UV-C inactivation studies

In the conduct of UV-C inactivation studies, 2.5 mL of acclimatized *S. enterica* cocktail cell suspensions were pipetted to 247.5 mL coconut liquid endosperm to introduce an initial population of 8.0 log CFU/mL. The inoculated medium was placed in a sterile 1.4-L glass loaf dish (21 cm × 11.6 cm), which was then placed in a fabricated UV-C box with a 15 W UV-C lamp (Sankyo Denki, Tokyo, Japan) that predominantly emits a 254 nm UV-C radiation. To confirm the predominant radiation emitted by the UV-C lamp in the fabricated box, the source was previously subjected to optical emission spectroscopy at a treatment distance similar to that used in this study (Fig. 1). Emission measurement was conducted using a spectrometer (Ocean Optics, Inc., Florida, USA) with a dispersion of 0.25 nm per pixel, and an optical resolution of 1.09 nm in the range of 200–1100 nm. Furthermore, per product specifications, the UV-C lamp used in this study had an irradiance value of 0.05 mW/cm^2 at a lamp-to-surface distance of 100 cm. Thus, using the inverse square relationship between irradiance from a point source and distance (Ryer, 1997), the irradiance received by the coconut liquid endosperm surface at a lamp-to-treated surface distance of 8.8 cm employed in this study was calculated to be 6.46 mW/cm^2.

Turbulent flow was introduced to the UV-C irradiated coconut liquid endosperm using a magnetic stirrer (HS1, Torrey Pines Scientific, California, USA) set at maximum rotational speed of 1500 rpm. After a predetermined UV-C exposure time, 1-mL aliquot was obtained from the treated medium for survivor enumerations. *Salmonella enterica* populations were determined by subjecting withdrawn treated samples to 10-fold serial dilutions in 0.1% peptone water (Peptone Water, HiMedia, Mumbai, India). Aliquots of 0.1 mL were withdrawn from appropriate dilutions and then surface-plated onto pre-solidified NA plates, prior to incubation at 37 °C for 24–48 h. Populations were enumerated 24–48 h post incubation, and were expressed as log CFU/mL. Two independent runs with two technical replicates per run were conducted for this phase of the study.

2.6. UV-C inactivation parameters

In this study, inactivation parameters were determined for the UV-C-treated *S. enterica* in coconut liquid endosperm. The $D_{UV\text{-}C}$ value of the test organism refers to the UV-C energy dose (mJ/cm^2) necessary to reduce the inoculated *S. enterica* population in the coconut liquid endosperm by 1 log cycle. The $D_{UV\text{-}C}$ value was determined as the negative inverse of the slope of the best-fitted inactivation curve interpolated by linear regression from the surviving population vs. exposure energy plot. In this study, $D_{UV\text{-}C}$ values were only calculated from inactivation curves that traversed at least 1 log cycle and with R^2 values not less than 0.9. Finally, to determine whether previous exposure to stresses resulted in heterologous adaptation to UV-C, the $D_{UV\text{-}C}$-ratio ($D_{UV\text{-}C,\ stressed}/D_{UV\text{-}C,\ control}$) in each of the treatment was calculated. In this study, ratios greater than 1.0 were considered as indicators of heterologous adaptation.

2.7. Statistical analysis

Data obtained from all independently replicated experiments were subjected to one-way Analysis of Variance (ANOVA) using the software IBM SPSS Statistics 22 (SPSS, Inc., 2013, New York, USA). Duncan's Multiple Range Test (DMRT) was used as post-hoc analysis whenever a significant difference.

3. Results and discussion

Food process schedules should be based on the inactivation rates of a predetermined resistant strain, species, or physiology of a reference organism, as this will provide a greater margin of safety towards less resistant contaminants (Koutchma, 2014; Koutchma, Forney, & Moraru, 2009). In this study, physiological state refers to the metabolic or structural state of the tested organism immediately after exposures to specific individual or simultaneous multiple stresses. *S. enterica* with varying physiological states were inactivated in UV-C treated coconut liquid endosperm. The suspending medium was chosen due to its sensitivity towards high temperature processing, and therefore a raw material that could potentially be processed by UV-C irradiation. Furthermore, coconut liquid endosperm has relatively higher pH (5.00) and low sugar content (5.1°Brix), which could not further contribute to physicochemical stresses on the target cells, and affect the subsequent UV-C resistance or susceptibility.

3.1. Sublethal injury

In this study, sublethal injury was determined as the fraction of the population exposed to stresses that can form colonies on the

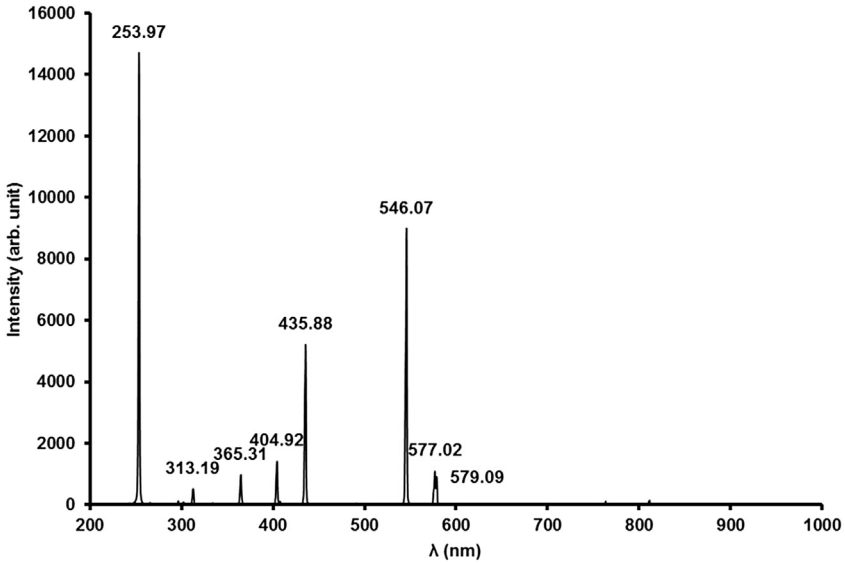

Fig. 1. Emission spectra of the 15-W UV-C lamp source showing predominant emission wavelength at 254 nm.

non-selective NA, but not on the selective BSA. Table 1 summarizes the varying rates of sublethal injury in the cocktail of 7 *S. enterica* strains after exposures to different physicochemical stresses. Among the stressed cells, the highest percentage was observed after exposure to simultaneous acid and desiccation stresses (46%), followed by exposure to all three stresses (44%). It should be noted that no significant difference was observed in the mean injury rates of all stresses. Despite the similarities in the injury rates obtained after exposures to different stresses, one can surmise that the nature of injuries the cells acquired are different.

Wu (2008) explains that different food-processing stresses can induce different types and amounts of damage to different sites in a microbial cell, which also subsequently results in variations in cellular repair mechanisms. Exposure of microorganisms to acidic environments has been reported to result in disruptions of their biochemical processes such as enzyme functions and nutrient transport (Beales, 2004). Damages in the RNA and reduction in nuclease and coagulase activities were also reported to be some of the outcomes of exposure to adverse acidic environments (Przybylski & Witter, 1979; Zayaitz & Ledford, 1985). Exposure of microbial cells to high temperature and desiccation has been reported to result in cell wall damage of microorganisms, further leading to losses in vital cellular materials including ions such as Mg^{2+} and K^{+}, amino acids and peptides, and nucleic acids (García, Gómez, Condón, Raso, & Pagán, 2003; Wu, 2008). Jay, Loessner, and Golden (2006) also discussed that when microorganisms are subjected to sublethal heat and freezing, many individual cells undergo metabolic injury, which results in their inability to form colonies on selective media that uninjured cells can tolerate. It should also be noted that injury was also detected in the control, non-stressed cells, which involved propagation NB. The injury rate in the control was smallest at 16%. This observation is possible since cells were harvested in the mid-stationary phase of growth. In the stationary growth phase, cell population starts to taper due to limitations in substrate and nutrients (Kolter, Siegele, & Tormo, 1993).

3.2. UV-C inactivation behavior of stressed *S. enterica* in coconut liquid endosperm

Fig. 2 illustrates the UV-C inactivation curves of *S. enterica* cocktails subjected to various singular and combined simultaneous physicochemical stresses in coconut liquid endosperm. After

Table 1
UV-C inactivation of stressed *Salmonella enterica* in coconut liquid endosperm (5.0 pH, 5.1°Brix, 0.15% malic acid, 14.3 mm sample thickness).

Stress Factors[a]	Growth Conditions				% Injury[d]	D_{UV-C} (mJ/cm^2)[e]	R^2 Range	
	pH[b]	a_w[c]	Temp (°C)	Time (h)			Min	Max
Control	7.0	0.99	37	24	15.7 ± 6.7 [a]	31.5 ± 2.7 [cd]	0.985	0.997
Acid (A)	4.5	0.99	37	24	21.1 ± 2.4 [a]	40.1 ± 2.1 [ab]	0.986	0.998
Desiccation (D)	7.0	0.96	37	24	18.6 ± 6.7 [a]	30.8 ± 1.8 [cd]	0.980	0.991
AD	4.5	0.96	37	24	46.0 ± 5.6 [a]	36.2 ± 7.1 [bc]	0.906	0.996
Heat (H)	7.0	0.99	40	24	25.0 ± 15.2 [a]	43.8 ± 4.1 [a]	0.903	0.995
AH	4.5	0.99	40	24	25.5 ± 6.7 [a]	40.3 ± 1.8 [ab]	0.984	0.993
DH	7.0	0.96	40	24	22.1 ± 0.1 [a]	29.8 ± 3.9 [d]	0.906	0.995
ADH	4.5	0.96	40	24	43.7 ± 17.5 [a]	22.5 ± 1.5 [e]	0.977	0.997

[a] *Salmonella enterica* serovars were individually exposed to physicochemical stress factors by propagating the cells at suboptimal growth conditions. Stresses: N = control; A = acid stress; D = desiccation stress; H = heat stress.

[b] Final pH after 24 h incubation. For acid stress exposure, gradual acidification occurred as a result of cellular assimilation of 1% w/v glucose supplemented in the nutrient broth growth medium, which resulted in the gradual liberation of organic acid metabolites.

[c] a_w of the nutrient broth growth medium. For desiccation stress exposure, the a_w of the medium was modified by adjusting NaCl content to 7.0%.

[d] Sublethal injury is the difference in the populations enumerated in the non-selective growth media that allows injury repair and eventual colony formation of injured and uninjured cells, and selective growth media that only allows colony formation of uninjured cells. Values followed by the same letter are not significantly different ($P < 0.05$).

[e] D_{UV-C} is the amount of UV-C energy necessary to reduce the initial population of *S. enterica* by 1 log cycle or 90%. Reported values are averages of four readings obtained from two independent runs. Values followed by the same letter are not significantly different ($P < 0.05$).

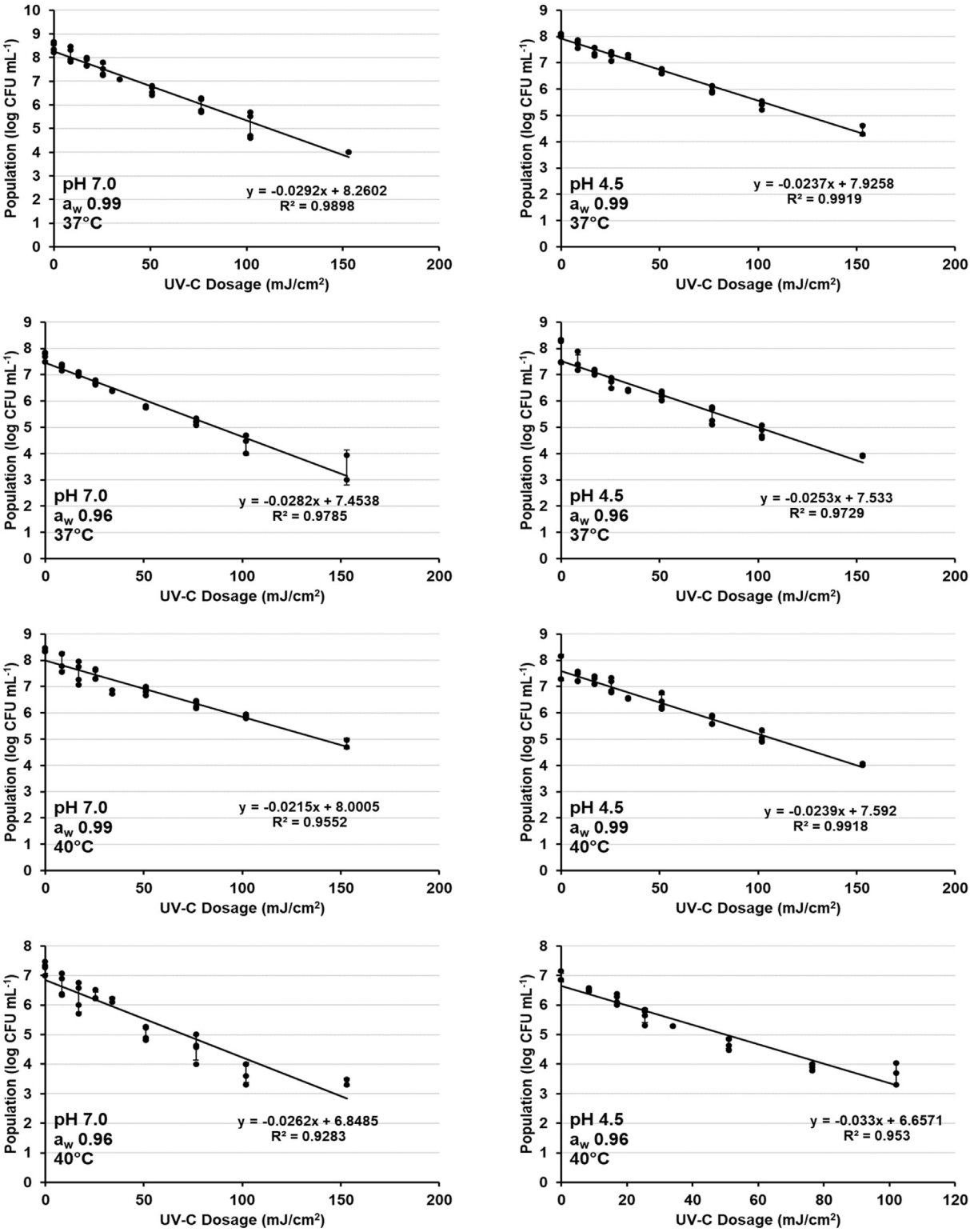

Fig. 2. Ultraviolet-C inactivation curves of cocktails of *Salmonella enterica* serovars in coconut liquid endosperm (5.0 pH, 5.1°Brix, 0.15% malic acid). Test organisms were subjected to propagation in various combinations of suboptimal growth conditions: control: pH ~7.0, a_w = 0.99, 37 °C, 24 h; with acid stress (gradual acidification): final pH ~4.5; with desiccation stress: a_w = 0.96; with heat stress: 40 °C.

exposures to all stresses, cells exhibited logarithmic linear inactivation behavior with R^2 values as high as 0.999. A logarithmic linear decline in the population is a hallmark of a first-order inactivation behavior that suggests homogenous susceptibilities of the different strains in the *S. enterica* cocktail treated with UV-C. This inactivation behavior was earlier reported by Moats (1971) for cells exposed to high temperature. Gabriel (2012a) similarly reported such linear trends for individual and cocktailed strains of *Escherichia coli* O157:H7 in a clear apple juice. Gabriel, Aguila, and Tupe (2015) reported linear logarithmic UV-C inactivation behavior for

acid- and desiccation-stressed *S. enterica* cocktailed serovars in a beverage composed of a mixture of young and mature coconut liquid. For young coconut liquid endosperm, Gabriel (2015) reported analogous UV-C inactivation behavior for cells previously exposed to separate and sequential acidification as well as desiccation stresses. Similar linear logarithmic UV-C inactivation behavior were also observed and reported for cocktailed *S. enterica* serovars previously exposed to individual and dual combinations of heat, acid and desiccation stresses in an orange juice system (Gabriel, Estilo, Isnit, & Membrebe, 2016).

It is commonly believed that microbial mortality as a result of exposure to a lethal step is a process following 1st order kinetics (Peleg, 2000). Consequently, the number or fraction of survivors decays exponentially with time and the existence of some survivors is inevitable, even after a long exposure time. However, there are also studies wherein non-linear inactivation behavior was observed (Bialka, Demirci, & Puri, 2008; Craik, Weldon, Finch, Bolton, & Belosevic, 2001; Marquenie et al., 2002; Unluturk, Atılgan, Baysal, & Tarı, 2008). Deviations from linearity include shoulders, tails or both. Moreover, distributions can also be wide or narrow, symmetric or asymmetric with a positive or negative skewness, and unimodal or bimodal. Peleg (2000) explains that different microorganisms have different survival distribution curves mainly because of processes and interactions at the molecular and cellular levels. According to Koutchma (2014), shoulder can be explained by DNA damage and repair phenomena. As for tailing behavior, this can be attributable to the UV-C resistance heterogeneity among populations, changes in susceptibility during treatments that can be explained by adaptation, and/or cell aggregation. For non-linear survival curves, the Weibull model is frequently used to describe the concave or convex microbial behavior (Koutchma, 2014; Peleg, 2000; van Boekel, 2002).

3.3. Effects of stresses on $D_{UV\text{-}C}$ value

In this study, results show that the $D_{UV\text{-}C}$ values of stressed *S. enterica* cells ranged from 22.5 to 43.8 mJ/cm^2 (Table 1). Cells were most resistant to UV-C inactivation when previously exposed to singular heat stress, and least resistant when simultaneously exposed to acid, desiccation, and heat stresses prior to UV-C treatment. While exposures to acid, acid + desiccation, and acid + heat stresses generally resulted in increased resistance against UV-C, exposures to desiccation and desiccation + heat stresses resulted in decreased resistance. The $D_{UV\text{-}C}$ values from heat stress were not significantly different ($P > 0.05$) from acid (40.1 mJ/cm^2) and acid + heat (40.3 mJ/cm^2) stresses, but were significantly different ($P < 0.05$) from all other stresses.

In the farm-to-fork model presented by Gabriel (2014), various physicochemical stresses, such as acidification, desiccation, or heat-induced stresses, may be individually or simultaneously encountered by microorganisms in raw materials and raw material production environments; which may bring about adaptive mechanisms in microorganisms that can influence their susceptibility towards the kill step during processing. As pathogens are naturally exposed to several stresses that may individually or simultaneously affect their behavior in foods, it is imperative to study the effects of not just one, but also several relevant stress factors on microbial inactivation and survival in food systems (Skandamis, Yoon, Stopforth, Kendall, & Sofos, 2008; Tiganitas, Zeaki, Gounadaki, Drosinos, & Skandamis, 2009). Common physicochemical stresses encountered by microorganisms in foods include changes in pH, either due to intrinsic food components or due to deliberate pH manipulations in food and food processing environments. Increased osmotic pressure due to reduction of a_w may also be encountered by microorganisms when subjected to drying conditions or increasing solute concentrations.

Lin, Lee, Frey, Slonczewski, and Foster (1995) explained that *S. enterica* and Enterobacteriaceae may encounter sublethal acid stress in the natural host environments as well as during passage through the stomach into the intestine. Excrement and other defecated materials are known to exhibit sublethal acid stress, and possibly heat stress to enteric microorganisms during the fermentation of fecal matter. A combination of desiccation and heat stress may also be induced from substrate dehydration. Indeed, several studies have shown that exposures of cells to sublethal stresses such as acidification, desiccation, temperature fluctuations, and their combinations could lead to cross protection from heat inactivation (Buchanan & Edelson, 1999; Gabriel & Nakano, 2011; Gabriel, 2012b; Mazzotta, 2001; Ryu & Beuchat, 1998; Sharma, Adler, Harrison, & Beuchat, 2005).

There are very few studies on the effects of prior exposures to environmental stresses on the subsequent UV-C susceptibility or resistance of microorganisms in food. The dearth in information regarding this phenomenon must be addressed, as efficacy of UV-C process schedules should be based on the inactivation rates of resistant species, strains, or physiological states of a reference organism. A recent study by Gabriel (2015) reported similar observations after *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* were similarly exposed to stresses prior to UV-C inactivation in coconut liquid endosperm. Increase in UV-C resistance was observed after exposure to the same gradually acidifying condition as well as exposure to sequential desiccation stress ($a_w = 0.85$) after acid stress exposure. For *S. enterica*, non-stressed cells were reported to have a $D_{UV\text{-}C}$ of 8.6 mJ/cm^2, while cells exposed to acid stress, and combined acid and desiccation stresses were reported to have $D_{UV\text{-}C}$ values of 20.5 mJ/cm^2 and 19.2 mJ/cm^2 respectively.

The disparities in the UV-C inactivation parameters observed between those of Gabriel (2015) and those reported in this study may be attributed to a number of variables including experimental setup in sample volume, lamp-to-surface distance, and flow properties, *e.g.* laminar vs. turbulent flowing. Even slight variations in the physicochemical properties of the suspending medium can influence microbial inactivation. When comparing inactivation parameters, emphasis should be given on the difference in sample volume and height as UV-C penetration in juices is relatively small, with 90% of radiation absorption limited to the first few milliliters (Franz, Specht, Cho, Graef, & Stahl, 2009; Sizer & Balasubramaniam, 1999). For this study, UV-C inactivation was carried out with constant stirring using a magnetic stirrer to induce turbulent flow in the sample. In doing so, this addresses the concern of uneven distribution of UV-C radiation in thick-layered UV-absorbing liquid products (Koutchma et al., 2009). Guerrero-Beltran and Barbosa-Canovas (2004) reported that, in order to ensure an adequate reduction of 5 log cycles of a surrogated microorganism and hence obtain a microbiologically safe food product, it is necessary for all parts of the fluid to be exposed to at least 40 mJ/cm^2 of UV light at 254 nm, considering that such dose should be applied to the entire food system to ensure that the liquid food is treated equally. Furthermore, it should be noted that in the previous work reported by Gabriel (2015), stresses were applied sequentially, not simultaneously like what was done in this recent work.

Gabriel et al. (2016) also studied the effects of prior exposure to physicochemical stresses on the subsequent susceptibility of *S. enterica* cells to UV-C in orange juice (pH 3.1, 11.5°Brix, 0.63% citric acid). Their results showed that heterologous adaptation towards UV-C irradiation take place after previous exposures to physicochemical stresses. The greatest resistance was observed when cells were previously exposed to desiccation stress (83.2 mJ/cm^2), and interestingly, the least resistance resulted from cells previously

exposed to heat stress (59.7 mJ/cm^2). The values reported by Gabriel et al. (2016) were however, not significantly different. The considerable disparities between the results of UV-C inactivation in orange juice and the results obtained in this study using coconut liquid endosperm (4 mL) may be attributed to a number of variables including experimental setup in sample volume, lamp-to-surface distance, and flow properties, i.e., laminar vs. turbulent flowing. The range of D_{UV-C} values calculated from this study are roughly half compared to those of Gabriel et al. (2016).

Aside from the differences in the volumes treated in Gabriel et al. (2016) and in this current study, the results obtained from this work suggest that the influence of prior stress exposure on the subsequent resistance or susceptibility of microorganisms are also dependent on the properties of the medium in which they are suspended. The differences in the physicochemical properties of orange juice used by Gabriel et al. (2016) and coconut liquid endosperm used in this study may have similarly contributed to the observed variations in the inactivation parameters. Gabriel (2012b) reported that the cross protection towards heating rendered by exposure to gradual acidification to *E. coli* O157:H7 was only observed in suspending medium with soluble solids <55°Brix.

3.4. Heterologous adaptation

The calculated D_{UV-C} ratio values (Fig. 3) showed that cells previously exposed to single acid and single heat, and combined acid + desiccation and acid + heat stresses exhibited heterologous adaptation towards UV-C irradiation. Different stresses that may be encountered by cells along the food production pipeline can induce different types and amounts of damage to varying sites in a microbial cell that also consequently results in variations in cellular repair mechanisms (Wu, 2008). Stress-induced microbial responses may include genetic and physiological changes that may enhance survivability (Foster & Hall, 1990; Goodson & Rowbury, 1989) against the same stress (homologous adaptation) (Tsakalidou & Papadimitriou, 2011) or an entirely different one (heterologous adaptation) (Al-Nabulsi et al., 2011; Buchanan & Edelson, 1999; Mazzotta, 2001; Sharma et al., 2005). Among those that exhibited heterologous adaptation, only cells previously exposed to the combined acid + desiccation stresses had resistance (15% more resistant) not significantly different ($P > 0.05$) from the control. Exposure to single heat stress resulted in the greatest heterologous adaptation, with cells being 39% more resistant to UV-C than the control.

Gabriel (2015) also reported adaptation and increased resistance to UV-C of *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes* after single and sequential exposures to acid and desiccation stresses. Sequential exposures to acid and desiccation stress generally resulted in the highest UV-C resistance for each test organisms. Gabriel et al. (2016) determined the effects of single and combined physicochemical stress exposures on the subsequent UV-C resistance of *Salmonella* spp. in orange juice. A general heterologous adaptation behavior towards UV-C was observed after exposure to almost all stresses except heat. Desiccation stress was reported to result in the most UV-C resistant cells; combinations of desiccation with other stresses were also reported to yield relatively UV-C resistant cells.

Similar to the trend observed in this current study, Mitchel and Morrison (1983) demonstrated that ultraviolet light resistance (up to 30 mJ/cm^2) was induced in *Saccharomyces cerevisiae* after heat-shocking at 36 or 38 °C. It has been reported that exposure of cells to rapid increase in temperature has been known to produce dramatic changes in the pattern of protein synthesis, usually inducing the synthesis stress or heat shock proteins that may be responsible of protecting the organisms from heat or other factors (Trautinger, Kindås-Mügge, Knobler, & Hönigsmann, 1996). It was further explained that the increase in resistance was due to the induction of the recombinational repair system and independent of the DNA excision repair process (Mitchel & Morrison, 1983). Pardasani and Fitt (1989) also reported a similar observation for *E. coli* JE1011 (UV resistance up to 6 mJ/cm^2) after transferring growing cells from 30 to 42 °C for 45 min. However, another strain *E. coli* B was reported to become more sensitive to irradiation yet more heat resistant after the same treatment. It was concluded that

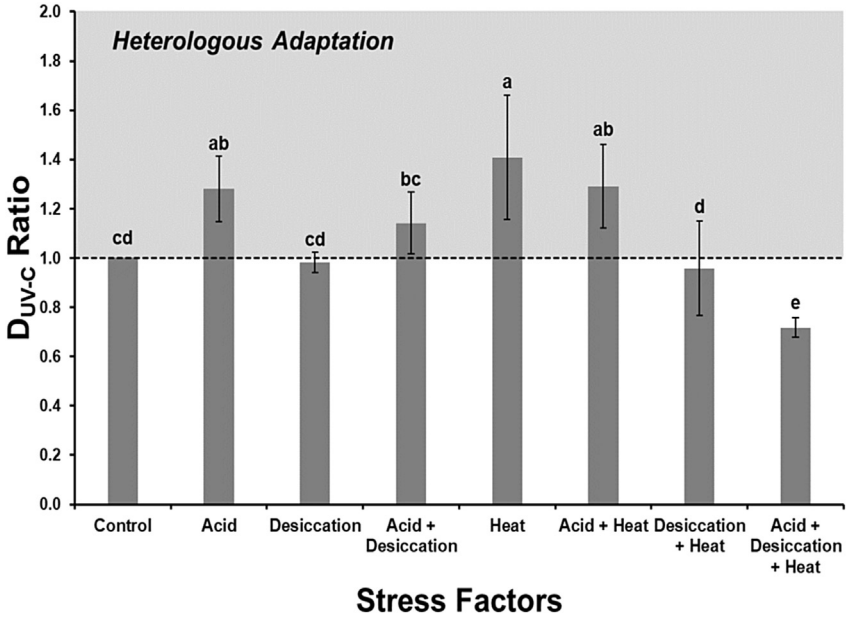

Fig. 3. Heterologous adaptation to UV-C in coconut liquid endosperm (5.0 pH, 5.1°Brix, 0.15% malic acid) after exposure to several physicochemical stresses. Test organisms were subjected to propagation in suboptimal growth conditions from single to cumulative combination stresses. Control: pH ~7.0, a_w = 0.99, 37 °C, 24 h; with acid stress (gradual acidification): final pH ~4.5; with desiccation stress: a_w = 0.96; and with heat stress: 40 °C.

ultraviolet resistance and thermal resistance are not induced together in these two strains and may arise by independent mechanisms (Pardasani & Fitt, 1989).

This study established the influences of selected implicit microbial characteristics of cell physiological states on the UV-C inactivation rates of *Salmonella enterica* in coconut liquid endosperm. The effects of microbial cell physiological state on UV-C resistance were determined by subjecting a composite of 7 strains of *S. enterica* subjected to individual and all possible combinations of gradual acidification, abrupt desiccation, and abrupt heat stress. Post stress exposure measurement of cell populations on selective and non-selective media confirmed that the suboptimal conditions to which the cells were exposed induced sublethal injury in cells. Results however, show that the magnitudes of % injury rates in cells subjected to all stresses were not significantly different. The study only used the number of cells not able to produce colonies on a selective medium as basis of sublethal injury. There is a need to characterize the nature of the injury a cell acquires after exposure to a specific stressful condition. The presence of stress-specific injury may be inferred from the inactivation rates of the stressed cells in UV-C treated young coconut liquid endosperm.

4. Conclusions

In this study, despite the multi-strain inoculum used as challenge organism, the cells exhibited first-order, linear logarithmic inactivation behavior, indicating the homogeneity in the resistance or susceptibility of cells to UV-C. However, despite the uniform inactivation behavior across stress types, the inactivation rates expressed as $D_{UV\text{-}C}$ values significantly varied. Heterologous adaptive mechanisms were observed after *S. enterica* cells were exposed to acid, acid + desiccation, heat, and acid + heat, with individual heat stress exposure resulting in the significantly most UV-C resistant cells. The apparent lack of relationship between % injury and inactivation rates may also be attributed to the fact that the natures of the injuries were not studied. Future works involving biochemical and structural characterization of cells post stress exposure may give light on the mechanisms that could explain increase in resistance of *S. enterica* towards UV-C after stress exposures.

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