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ORIGINAL ARTICLE

Changes in cellular structure of heat-treated *Salmonella* in low-moisture environments

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Abstract

Aims: *Salmonella* cells desiccated in an environment with low-water activity (a_w) show longer survival times and enhanced resistance to heat. However, little is known about the cellular ultrastructure of *Salmonella* in low-a_w environment in relation to the survival and persistence during desiccation.
Materials and Results: In this study, *Salmonella* Enteritidis strain PT30 was dehydrated by exposure to air or by mixing with wheat flour (a_w 0·30 at room temperature) for 7 days followed by heat treatment at 80°C for 10, 20, 60 min respectively. Transmission electron microscopy (TEM) was employed to examine and compare the ultrastructure of heat-treated *S.* Enteritidis cells after desiccation with the cells suspended in trypticase soy broth (TSB). Cells suspended in TSB broth showed disrupted ribosomes, congregated proteins and denatured DNA. However, no significant alterations were observed in the ultrastructure of the desiccated cells after heat treatment. The number of desiccated *S.* Enteritidis cells decreased by <1·5 log CFU per gram after 80°C treatment for 60 min, however, cells suspended in TSB declined more than 5 $\log_{10}$ CFU per mL at 80°C within 5 min.
Conclusions: A drastic difference in the number of survivors and cellular ultrastructure was observed between vegetative and air or food-dried *S.* Enteritidis cells after subjecting to heat treatment at 80°C. No significant ultrastructure changes were observed in desiccated cells after heat treatment except for roughening and corrugating surfaces.
Significance and Impact of the Study: This study provides a direct comparison to illustrate how desiccation influences the cell ultrastructure before/after heat treatment, which will aid in better understanding of the fundamental mechanism underlying the increased thermal resistance of *Salmonella* cells in low-a_w environment.

Highlights

- Desiccated *S.* Enteritidis cells have enhanced survival and persistence to heat.
- TEM imaging visualizes the cell structure changes during heat treatments.
- Nondesiccated cells after heat treatments have severe damage in cellular ultrastructure.
- Heat-induced ultrastructure damage of desiccated cells was not obvious.

Introduction

Water is a major component of most living organisms and usually comprises more than 80% of the total cell mass (Bratbak and Dundas 1984). Micro-organisms rely on water for growth and no bacteria can propagate when water is absent. Water activity (a_w), defined as the ratio of the water vapour pressure of the food to the vapour pressure of pure water at the same temperature (Rockland and Beuchat 1987), represents a physicochemical

parameter that quantifies the link between the amount of moisture available in foods and the ability of micro-organisms to perform metabolic reactions for growth (Scott 1958). Most bacteria cannot grow below a_w of 0·85, however, they can survive at low a_w (Beuchat *et al.* 2013). For instance, the minimum a_w growth level for *Salmonella* is 0·93, but they can adapt and survive for long periods in low-a_w food matrices (Hiramatsu *et al.* 2005; D'Aoust and Maurer 2007; Finn *et al.* 2013; Podolak and Black 2017), such as almond kernels (Uesugi *et al.* 2006), raw walnut (Blessington *et al.* 2012) and dried milk products (Ray *et al.* 1972).

The safety of low-moisture foods (LMFs) has gained a lot of attention in recent years with multiple outbreaks due to food-borne pathogens, such as *Salmonella.* Low-moisture products were previously considered safe because of the assumption that the low-a_w environment would not support the growth of bacterial pathogens. Consequently, the products are packaged without additional pathogen inactivation steps. However, the risk of food-borne illnesses including Salmonellosis significantly increases if such products are not properly cooked or consumed raw (e.g. cookies dough).

Salmonella is the most intensely studied bacteria for its enhanced thermal resistance in low-a_w food systems (Gruzdev *et al.* 2011). For example, nondehydrated *S.* Typhimurium LT2 cells do not survive when subjected to heat treatment at 135°C for 30 min. However, when *S.* Typhimurium LT2 cells are dehydrated on hydrophobic membranes at 57% equilibrium relative humidity (RH) for 48 h at 37°C prior to heat treatment at 135°C for 30 min, significant increase in the survival of *Salmonella* was reported (Kirby and Davies 1990). In low a_w environment, *Salmonella* also exhibits cross-tolerance to other stresses including chemical (ethanol, sodium hypochlorite, sodium chloride, etc.) and physical (such as UV irradiation) treatments (Gruzdev *et al.* 2011; Finn *et al.* 2013). The *D*-values (the time in minutes to kill 90% of target bacteria at a specific temperature) of *Salmonella* increase exponentially with decreasing a_w at a given treatment temperature (Liu *et al.* 2018b; Xu *et al.* 2018b). It is postulated that the reduced a_w in LMFs exerts a protective effect against thermal inactivation of *Salmonella* in food products (Burnett *et al.* 2000; Mattick *et al.* 2003; Kieboom *et al.* 2006; Van *et al.* 2013; Zhang *et al.* 2018). However, the mechanism underlying *Salmonella* survival and persistence in LMFs is poorly understood. Previously, Deng *et al.* (2012) reported that <5% of the genome of *Salmonella* is transcribed in a desiccated state in peanut oil (a_w = 0·3 at room temperature), suggesting that *Salmonella* cells largely remain in a physiologically dormant state in this environment. Deng *et al.* (2012) suggested that DNA protection and noncoding RNAs play roles in *Salmonella* desiccation response. Increased expression of heat shock proteins (Hsp70) and its association with cellular subfractions likely protects against heat damage in low-a_w-environment (Kampinga *et al.* 1995).

Till today, most of the research has focused on quantitative measures of the survivors and thermal resistance of *Salmonella* cells in a low-a_w environment (Villa-Rojas *et al.* 2013; Liu *et al.* 2018a; Xu *et al.* 2018b). The effect of desiccation on the thermal resistance of *Salmonella* cells in terms of cell structure has not been systematically investigated (Gruzdev *et al.* 2011; Maserati *et al.* 2017). The objective of this study was to determine the ultrastructure of *Salmonella* in low-a_w environment and its impact on survival and persistence during thermal treatments after desiccation. Our hypothesis was that desiccated *Salmonella* cells resulted from low-a_w environment are more resistant to overall heat-induced damage to the cellular ultrastructure than nondesiccated cells (i.e. cells in high-a_w environment). We used transmission electron microscopy (TEM) to study the ultrastructure morphology (e.g. cell envelope, ribosomes, nucleic acid) of heat-treated *Salmonella* cells after desiccation in air or low-a_w food environment (wheat flour) under the 10% RH at room temperature (~20°C).

Materials and methods

Culture preparation

The strain investigated in this study was *Salmonella enterica* sub sp. *enterica* serovar Enteritidis phage type PT30 (*S.* Enteritidis ATCC BAA-1045). This strain was isolated from contaminated raw almonds (Isaacs *et al.* 2005; Anderson *et al.* 2013; Podolak and Black 2017). *Salmonella* Enteritidis stock culture was maintained in trypticase soy broth supplemented with 0·6% (w/v) yeast extract (TSBYE) and 20% (v/v) glycerol at −80°C. A loopful of *S.* Enteritidis cells from the stock was inoculated into 9 ml TSBYE and incubated at 37°C for 24 h followed by plating of 1 ml culture on trypticase soy agar supplemented with 0·6% (w/v) yeast extract (TSAYE) plate (150 × 15 mm). After incubation at 37°C for 24 h, bacterial lawns were harvested from the plates with 10 ml of 0·1% (w/v) peptone water and centrifuged at 6000 ***g*** at 4°C for 15 min. After discarding the supernatant, the bacterial pellets were washed with 0·1% (w/v) peptone water for at least two times and resuspended in 3 ml of 0·1% (w/v) peptone water at 20°C to a cell density of ~10 $\log_{10}$ CFU per ml.

Salmonella Enteritidis cells were prepared under three different conditions. These included (i) control cells (C): vegetative *S.* Enteritidis cells in a planktonic form as

described above, (ii) air-dried cells (AD): vegetative *S.* Enteritidis cells after 7-day desiccation at 20°C under 10% RH and (iii) food-dried cells (FD): vegetative *S.* Enteritidis cells after 7-day desiccation at 20°C in wheat flour under 10% RH. The wheat flour used in this study had an initial moisture content 8·18 gH_2O per 100 g sample and a_w 0·32 ± 0·05 measured at 20°C. In this study, the average particle size of wheat flour measured by an ATM sonic sifter (ATM Corporation, Milwaukee, WI) was 144 ± 60 μm. For the preparation of C and AD cells, 500 μl of vegetative *S.* Enteritidis cells containing 10·1 ± 0·1 $\log_{10}$ CFU per ml in planktonic form were transferred in presterilized 1·5 ml Eppendorf® safe-lock microcentrifuge tubes (500 μl per tube). For preparation of FD cells, different batches of 500 μl of vegetative bacterial culture were mixed thoroughly with 10 g of wheat flour and placed in a Petri dish at 20°C. Microcentrifuge tubes and inoculated wheat flour were placed in a desiccator under 10% RH for 7 days at 20°C to prepare AD and FD samples, separately. Cell counts of *S.* Enteritidis after desiccation was conducted for both AD and FD samples, and <1 $\log_{10}$ CFU per ml reduction of population numbers was observed.

Thermal treatment

To investigate the effect of desiccation on the thermal resistance of *S.* Enteritidis cells, bio-samples (C, AD and FD) were subjected to heating at 80°C and held at this temperature for 5, 10, 20 and 60 min (the initial density of C was ~10 $\log_{10}$ CFU per ml. C and AD cells were treated in 1·5 ml microcentrifuge tubes (Fisherbrand™ Sanp-Cap Flat-Top Tubes; Fisher Scientific Inc., Fair Lawn, NJ), while FD cells were treated in an aluminium thermal death test cell (TDT cell, sample weight 0·7 g, 4 mm in thickness) (Chung *et al.* 2008). Isothermal treatments were conducted by immersing microcentrifuge tubes with closed cap to avoid moisture loss and TDT cells in a well-stirred oil bath (Isotemp 5150 H11; Fisher 180 Scientific Inc., PA) preheated to 80°C. The come-up time for all bio-samples to reach the target temperature was <2 min. After the heat treatments, all samples were cooled down immediately in an ice-water bath for 3 min.

Enumeration

To obtain the viable counts of *S.* Enteritidis cells after the heat treatments, 1 ml of control sample was diluted in 9 ml of 0·1% (w/v) peptone water; AD samples were resuspended in 500 μl of 0·1% (w/v) peptone water; FD samples (0·7 g) were scrapped from test cells and transferred into 6·3 ml 0·1% (w/v) peptone water, to reach 10-fold dilution and centrifuged at 230 rev min^{-1} for 3 min (Stomacher® 400 Circulator; Seward Laboratory Systems Inc., Norfolk, UK). Next, 10-fold dilutions were plated in duplicate on TSAYE plate supplemented with 0·05% (w/v) ferric ammonium citrate (Sigma-Aldrich, St. Louis, MO), and 0·03% (w/v) sodium thiosulfate (Sigma-Aldrich) followed by incubation at 37°C for 48 h. Colonies with a dark centre and clear circle were identified as typical *S.* Enteritidis cells. The average number of survivors (CFU per ml) was based on three independent technical replicates of each sample for each treatment. The survivors were tested on the same day after heat treatment.

TEM

In total, 12 bio-samples from three treatment conditions corresponding to 0, 10, 20, 60 min heat treatment time respectively were examined by TEM. For fixing bio-samples, 100 μl of control samples (C0, C10, C20, C60), a small aliquot of AD (AD0, AD10, AD20, AD60) and FD (FD0, FD10, FD20, FD60) cells were transferred into 1·5 ml Eppendorf safe-lock microcentrifuge tubes with 2% (v/v) paraformaldehyde/2% (v/v) glutaraldehyde/acetone overnight at 4°C. The fixative was removed from the tube, and the substituted samples were washed with acetone for three times (each time 10 min). Samples were then transferred into 1% (w/v) osmium tetroxide in an acetone basis and frozen in liquid nitrogen for 1 h followed by rinsing thrice with acetone. Dehydration was performed by adding 0·5 ml acetone and 0·5 ml propylene oxide to the final bio-samples for 10 min. Dehydrated bio-samples were polymerized for 3 days at 75°C to form polyethylene capsule. Thin sections (50–70 nm) were cut with a glass knife on a Reichert Om U2 ultramicrotome (Leica, Bensheim, Germany). The sections were mounted on formvar coated copper grids, stained with 2% aqueous uranyl acetate and 0·1% lead citrate (Reynolds, 1963) and observed in Tecnai G2 20 TEM (FEI Company, Hillsboro, OR) at 200 kV acceleration voltage (Xu *et al.* 2018a). At least five different fields per bio-sample were captured at 5 K magnifications, and at least five different individual bacterial cells per bio-sample were imaged at 29 K magnification.

Statistical analysis

A two-way ANOVA (general linear model) was used to compare the estimated marginal means of *Salmonella* survivors under different treatments (C, AD and FD) at different heating time points (0, 5, 10, 20, 60 min). Statistical analysis was performed using SPSS software (ver. 22.0).

Results

Effects of dehydration on the survival of heat-treated *Salmonella* cells

After desiccation for 7 days under 10% RH at 20°C, the counts of AD cells decreased by 0·8 log, from 10·1 ± 0·1 $\log_{10}$ CFU per ml to 9·3 ± 0·1 $\log_{10}$ CFU per ml. On the other hand, FD cells decreased by 0·9 log, from 8·8 ± 0·1 $\log_{10}$ CFU per gram to 7·9 ± 0·1 $\log_{10}$ CFU per gram. This observation was consistent with a previous study indicating that most *Salmonella* strains declined <1 log after 4–5 days of equilibration in wheat flour at room temperature (Villa-Rojas 2015).

The number of *S.* Enteritidis survivors (CFU per ml) were significantly different ($P < 0·05$) between control cells (C) and dried cells (AD and FD), while no significant difference was observed between AD and FD. When control *Salmonella* cells were treated at 80°C, a 5·7 log reduction of liquid *S.* Enteritidis cells was achieved within 5 min (Fig. 1). This is not surprising because *S.* Enteritidis cells in an aqueous environment can be killed by thermal treatment quickly with an excess temperature of 74°C (Beuchat *et al.* 2013). However, both AD and FD cells showed enhanced resistance to heat with significantly more survivors after the thermal treatments, compared with the control (not detectable after 20-min treatment). For instance, after 60-min treatment, only 1·4 and 1·3 log reduction of *S.* Enteritidis cells was observed for AD and FD respectively (Fig. 1). The a_w of inoculated wheat flour after desiccation was 0·25 at room temperature, and the estimated *D*-value (~40 min) of *Salmonella* desiccated in wheat flour in this study was comparable with the previously reported *D*-value (46·8 ± 1·0 min) of *Salmonella* under the same treatment conditions at 80°C (Xu *et al.* 2018b).

Effects of dehydration on the ultrastructure of heat-treated *Salmonella* cells

For examination of the ultrastructure of *Salmonella* cells within heat-treated bio-samples, we first quantified the number of cells captured at 5 K magnification using Image J software (Rasband 1997). Next, we selected at least five representatives of *S.* Enteritidis cells from each bio-sample for ultrastructure examination at 29 K magnification (Fig. 2). Table 1 shows the total numbers of counted cells and the number of normal cells (single, separated complete cells with regular shape under TEM). In general, *Salmonella* cells shown in Fig. 3 are selected as representatives of 97% of the most commonly appearing *S.* Enteritidis cells in each bio-sample captured by TEM at 5 K magnification.

Effect of heat treatment at 80°C on the ultrastructure of *Salmonella* cells: Prior to heat treatment, the control *Salmonella* cells (Fig. 3, a1), showed smooth and continuous cell-envelopes (CE). The round and elongated images reveal inner structures in transverse and longitudinal (or sagittal) planes, respectively, of the sample cells. In these control cells, the margins of the cell envelope (CE), periplasmic region and cytoplasmic membrane are clearly identified with a homogeneous distribution of cytoplasmic proteins (CP) and electron-transparent nuclear material (n) in the cytoplasmic region. Within 10 min after

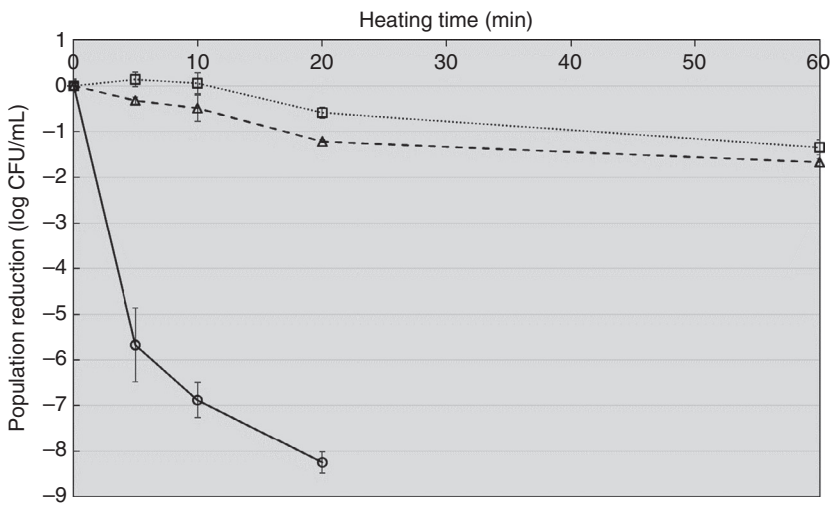

Figure 1 Profile plot of population reduction ($\log_{10}$ CFU per ml) of *Salmonella* Enteritidis cells after heat treatment (80°C) estimated by two-way ANOVA general linear model. The number of *S.* Enteritidis survivors (CFU per ml) of Control cells is significantly different ($P < 0·05$) than Air-dried and Food-dried cells. Each data points were calculated from three independent replicates ($n = 3$) (–⊖– Control; -▲- Air-dried; ··⊡·· Food-dried).

heat treatment at 80°C (Fig. 3, a2), control cells show accumulation of large protein aggregates (PA) within the cytoplasm. Such protein aggregates arise as a result of protein misfolding in response to stressful environmental changes (Bednarska *et al.* 2013). Denaturation of ribosomal subunits and soluble cytoplasmic proteins are more readilty denatured in heat treatments than DNA (Mackey *et al.* 1991). Moreover, particles forming the cylindrical arrays were identified as ribosomes due to their staining properties (Maniloff *et al.* 1965). Thus, the observed proteinic aggregates are likely ribosomal and other cytoplasmic proteins. During the 20- and 60-min treatment at 80°C (Fig. 3, a3, a4), control *Salmonella* cells showed changes indicative of aggregation of different regions of DNA (D) together with denaturation of CP (Mackey *et al.* 1991; Lee and Kaletunc 2002). The control *Salmonella* cells also show significant loss of viability at 10, 20 and 60 min post heat treatment (Fig. 1).

In contrast, to control *Salmonella* cells, air-dried cells did not show accumulation of large protein aggregates (PA), aggregation of DNA (D) or denaturation of CP (Fig. 3, b1–4) after heat treatment. Moreover, air-dried *Salmonella* cells had significantly increased survival as compared with the control *Salmonella* cells (Fig. 1). The air-dried cells showed healthy cell structure except for a little swollen in the endpoints which might have occurred because of the sample preparation (Fig. 3, b1). The surface of dehydrated cells was not as smooth as liquid cells. Nucleoplasm was very fine and indented with very sharp limits between nucleoplasm and cytoplasm. No significant differences in the image acquisition were noticed from air-dried *S.* Enteritidis cells after heat treatment at 80°C for 60 min. The distribution of electron densities was as nearly natural as could possibly be obtained with TEM for this sample type. We noticed membrane blebbing in some cells after 30-min heat treatment (see the highlighted region in b3). A large mesosomal element (M) was visible after 60-min heat treatment (Fig. 3, b4); the appearance of this mesosome-like structure may be the result of damaging the cytoplasmic membrane and cell wall (Balkwill and Stevens 1980).

The ultrastructure of food-dried *Salmonella* cells appeared largely stable with the accumulation of large protein aggregates starting after 20-min and 60-min heat treatments (Fig. 3, c3, c4), however other changes in the cell cytoplasm were not observed, except for induced membrane surface disruption (Fig. 3, c1) in comparison to the cells without drying (Fig. 3, a1). Intact cell membrane and intracellular contents were observed in food-

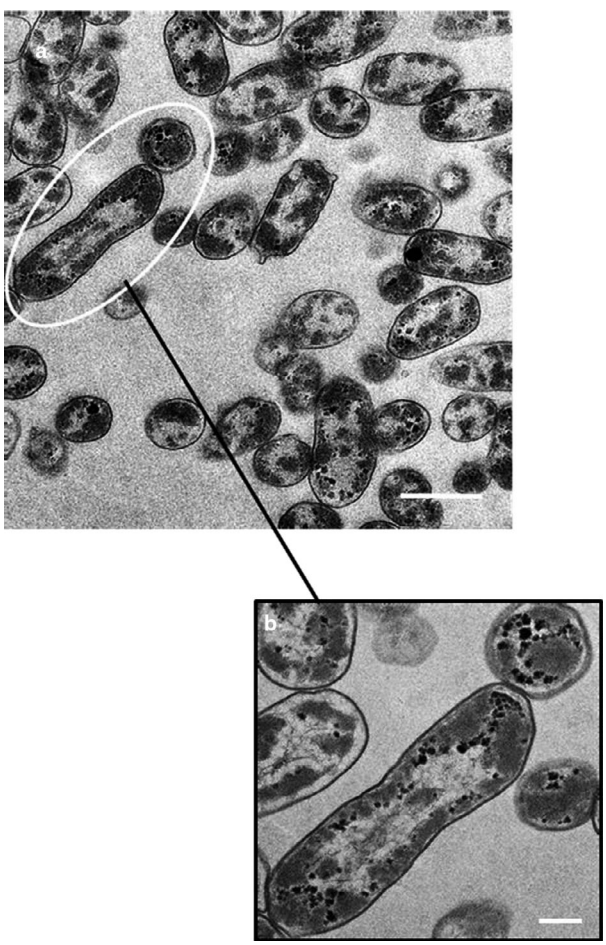

Figure 2 (a) A representation of *Salmonella* cells (heat-treated at 80°C for 10 min) captured at the 5 K magnification, scale bar = 1 μm; (b) the representative cell selected for visualization of ultrastructure at 29 K magnification, scale bar = 200 nm. The 5 K image was used to calculate the number of cells, and then a representative *S.* Enteritidis cell was captured at 29 K for ultrastructure analysis. A total of five representative cells were examined at 29 K for each bio-sample.

Table 1 Number of heat-treated *Salmonella* cells captured by TEM and the number of normal cells

Sample condition	Treatment time (min)	Number of cells		
		Examined	Normal	Normal (%)
Liquid control (C)	0	294	287	97·6
	10	615	598	97·2
	20	196	193	98·5
	60	222	218	98·2
Air-dried (AD)	0	207	204	98·6
	10	570	562	98·6
	20	138	134	97·1
	60	544	541	97·6
	0	54	53	98·2
Food-dried (FD)	10	17	17	100
	20	50	50	100
	60	24	24	100

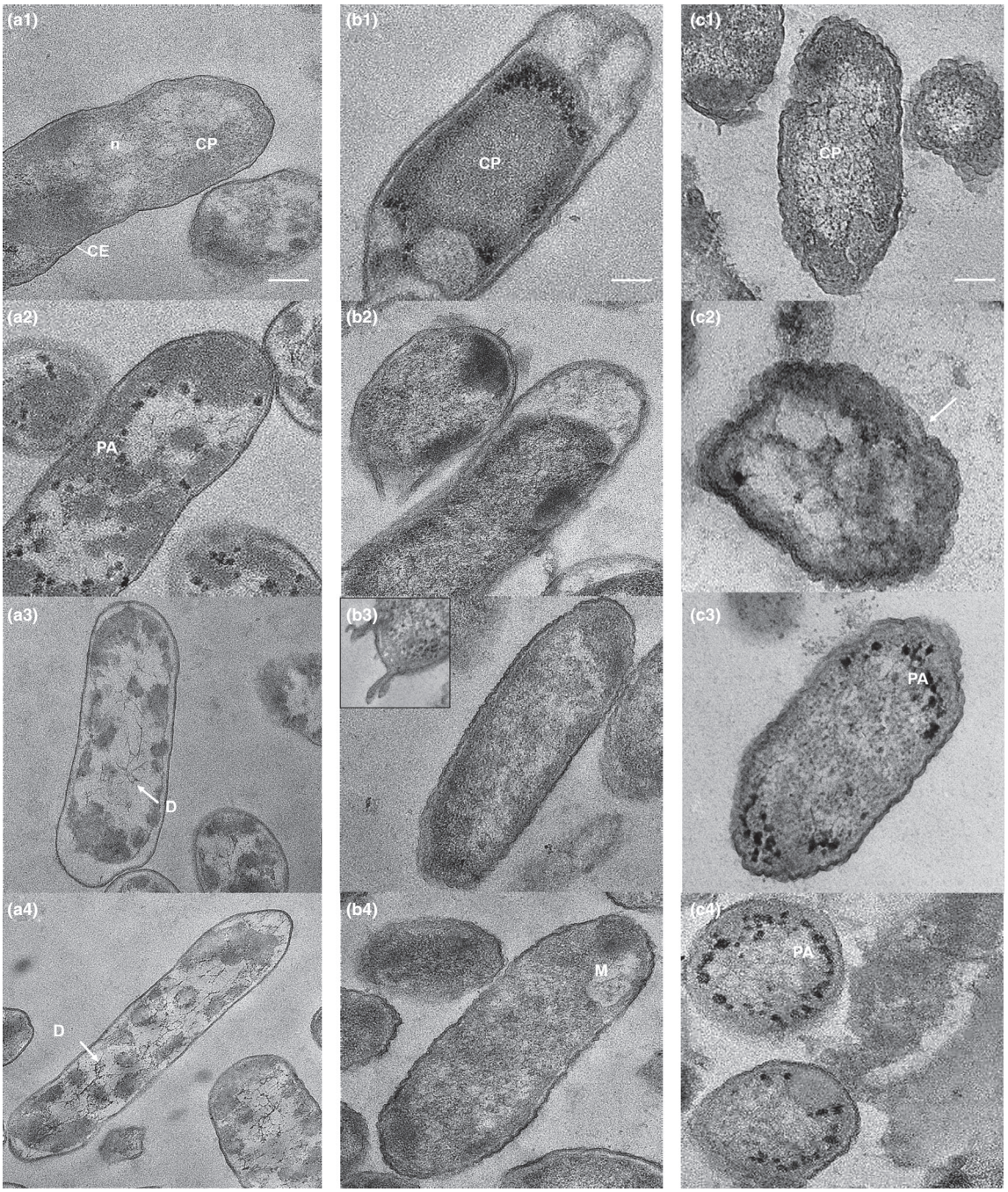

Figure 3 Representative transmission electron micrographs of thin-sectioned *Salmonella* Enteritidis PT30 control cells without drying (a), after air-drying (b), and after food-drying in wheat flour (c). The numbers 1–4 represents heat treatment for 0, 10, 20, 60 min at 80°C respectively. Scale bar = 200 nm. Plate a1 shows smooth and continuous cell envelopes (CE). Plates a1, b1–4, c1–4 show a homogeneous distribution of cytoplasmic proteins (CP) and electron-transparent nuclear material (n). Plates a2, c3–4 shows accumulation of large protein aggregates (PA). Plates a3 and a4 show DNA (D) aggregation and CP denaturation. Plate b3 and b4 show membrane blebbing (highlighted region) and a large mesosomal element (M) respectively.

dried *Salmonella* after 10-min heat treatment (Fig. 3, c1) with a typical membrane structure of severe corruption and undulation (arrow in Fig. 3, c2), indicating the influence of moisture loss or interaction with food particles on the cell surface structure. During the sample preparation, the long axis of the bacterial rods was mostly

positioned vertically to the spur's surface, and thus, the thin horizontal sections through a monolayer of bacteria always reveal the transversally cut bacteria. In this case, limited longitudinally cut bacteria were obtained as thin-sectioned samples. Therefore, only representative cross-sectional views were selected for heat-treated food-dried *S.* Enteritidis cells (Fig. 3, c2 and c4).

Discussion

Vegetative *Salmonella* cells can be readily destroyed by temperature treatments in high moisture environments due to disrupted ribosomes and congregated cytoplasmic region. Our results agree with previously published report wherein heat treatment of *E. coli* cells was reported to result in similar changes in cellular ribosomes and DNA and was also associated with the apparent loss of viability (Mackey *et al.* 1991; Lee and Kaletunc 2002). The degradation of ribosomal RNA as a result of heat treatment can be a direct cause of cell death as the absence of working ribosomes leads to the loss of protein synthesis to maintain essential functions (Tolker-Nielsen and Molin 1996). Thus, the mechanism by which thermal inactivation of control *Salmonella* cells that occurred in the treated liquid cells might include a significant reduction of 16S ribosomal RNA contained in the cells, unfolded and denatured protein, enzyme inactivation and even DNA damage (Nguyen *et al.* 2006; Wu 2008). However, after drying in the air or low-a_w foods (wheat flour), *S.* Enteritidis cells exhibited significantly enhanced resistance to heat with <1·5 log reduction after heat treatment at 80°C for 60 min. TEM images suggest that air-drying or food-drying of *Salmonella* cells somehow protects cytoplasmic proteins and DNA of *Salmonella* from heat-induced damage. In fact, the absence of water may have restricted the molecular mobility and protein deformation in living cells and that may explain why desiccated *Salmonella* cells show increased heat resistance (Potts 1994; Ball 2008). It is notable that *S.* Enteritidis cells generally maintained their rod shape after heat treatments. The cell wall which was apparently visible outside the cytoplasmic membrane was not leaky. No apparent collapse in the outer membrane of the bacterial cell was observed. This suggests that the high temperature at 80°C did not disintegrate the cell. We also noticed aggregation of food granulates around the *Salmonella* cells. Similar results were reported by Kuda *et al.* (2015). This phenomenon can be explained by the fact that carbohydrate compounds may increase the surface-adherent resistance of bacterial cells (*Salmonella*) during desiccation.

This is the first study that shows the differences in the ultrastructural changes of desiccated *S. Enteritidis* cells after heat treatment and provides an initial insight into the mechanism employed for long-time desiccation survival and thermal tolerance. Quantitative analyses are desirable in future studies to compare the cell size after desiccation and thermal treatment. More systematic studies are also needed to reveal the molecular mechanism of enhanced heat resistance of *Salmonella* cells in a low-moisture environment.

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Conflict of Interest

No conflict of interest exists in this manuscript. The work described in this manuscript is an original research that has not been published previously, in whole or in part.

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