



# The influence of temperature and water activity on thermal resistance of *Salmonella* in milk chocolate

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## ABSTRACT

*Salmonella* contamination of chocolate-derived products has caused several outbreaks and recalls in recent years. Earlier research found that reducing moisture content or water activity of low-moisture foods sharply enhances the resistance of *Salmonella* during thermal treatments. However, there is a lack of data that correlates the relationship between temperature, water activity ( $a_w$ ), and thermal resistance of *Salmonella* in milk chocolate. In this study, milk chocolate was inoculated with *Salmonella* Enteritidis PT 30 and conditioned to  $a_w$  of 0.23, 0.33, and 0.43 at room temperature ( $21 \pm 2^\circ\text{C}$ ). The chocolate samples were heated at 70, 75, and 80 °C to obtain the  $D$ -values (time to inactivate 90% of the test microorganisms at a given temperature) of *S. Enteritidis* PT 30. The change of  $a_w$  of milk chocolates at elevated temperatures (up to 80 °C) was also investigated. The results showed that the  $D$ -value of *S. Enteritidis* PT 30 decreased exponentially with the increase of  $a_w$  or temperature. The maximum  $D$ -value was  $47.4 \pm 3.7$  min obtained at 70 °C and  $a_w$  of 0.23. The minimum  $D$ -value was  $5.2 \pm 1.0$  min at 80 °C and  $a_w$  of 0.43. The  $z$ -values were found as the followings:  $z_{a_w, 70^\circ\text{C}} = 0.42$ ,  $z_{a_w, 75^\circ\text{C}} = 0.36$ , and  $z_{a_w, 80^\circ\text{C}} = 0.46^\circ\text{C}$ ;  $z_{T, 0.23} = 18.8 \pm 2.5$ ,  $z_{T, 0.33} = 20.6 \pm 4.1$ ,  $z_{T, 0.43} = 18.1 \pm 0.5^\circ\text{C}$ . Overall, the results from this research may provide useful information to help the industry control the risk of *Salmonella* contamination and improve microbial safety in chocolate production.

## 1. Introduction

The risk of *Salmonella* contamination in chocolate has been a concern for the food industry over the past several decades. In 1970, *Salmonella* Durham caused 110 cases of infection through contaminated cocoa powder, an ingredient used in chocolate products (WHO, 1973). In 1982, a *Salmonella*-contaminated chocolate bar (*Salmonella* Napoli) resulted in 245 infections in the U.K. (Gill et al., 1983). The most severe *Salmonella* outbreak (*Salmonella* Oranienburg) can be traced back to 2001 in Europe, where over 400 people were reported sick from consuming contaminated German chocolate (Werber et al., 2005). The latest *Salmonella* outbreak in chocolate products was reported by the European Centre for Disease Prevention and Control (ECDC) starting from the beginning of 2022. This outbreak has caused over 450 illnesses so far (by August 8, 2022) due to contamination of *Salmonella* Typhimurium (ECDC, 2022). Overall, there have been two types of contaminations in milk chocolate that can cause *Salmonella* outbreaks: 1) from raw ingredients, like cocoa beans, that were contaminated from harvesting or transportation (Komitopoulou & Peñaloza, 2009); 2) from the

processing plants of poor sanitary conditions. For example, it was reported by Hughes et al. (2008) that the *Salmonella* outbreak which occurred in the U.K. in 2006 was due to poor hygienic conditions.

Water activity ( $a_w$ ) of food is an important factor for the stability and growth of micro-organisms in a food product. Water activity can be defined as the ratio between the water vapor pressure of a food matrix and the water vapor pressure of pure water at the same temperature (FDA, 2014). In recent years,  $a_w$  has been often reported as a key factor in the thermal resistance of bacteria in low-moisture foods. For example, He et al. (2013) showed that an increased  $a_w$  (up to 0.8) of peanut butter reduced the thermal resistance of *Salmonella* in the sample at 90 and 126 °C. Earlier research documented the negative relationship between the moisture content of chocolate and the thermal resistance of *Salmonella* in the chocolate but did not correlate the result to  $a_w$  (Barrile, Cone, & Keeney, 1970). Moreover, there is a lack of general information concerning how  $a_w$  of milk chocolate changes with temperature in thermal treatments that are used for thermal control of *Salmonella*.

Chocolate ( $a_w$  0.3–0.5, moisture < 2%) is a low moisture food with high sugar and fat content (Krapf & Gantenbein-Demarchi, 2010).

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Roasting is the first thermal processing for cocoa beans harvested from the field. After that, conching is the only thermal treatment in chocolate processing (Toker, Palabiyik, & Konar, 2019). During conching, all ingredients are mixed and cross-contamination may happen in this step. The purposes of conching are to evaporate moisture, reduce particle size, mix all the ingredients and accelerate aroma release but pathogen reduction was not taken into consideration (Bolenz, Thiessenhusen, & Schäpe, 2003; Counet, Callemien, Ouwerx, & Collin, 2002; Keogh, Murray, Kelly, & O'Kennedy, 2004; Owusu, Petersen, & Heimdal, 2012). In the chocolate industry, it has been acknowledged that a low-temperature treatment is preferred over a high-temperature treatment for the best quality of the product. Lin (2010) noted that 70–80 °C treatment temperatures can promote Maillard reactions and release volatiles which may improve the quality of chocolate. Yet, depending on the desired quality of the final products, the conching temperature varies from 50 °C to 75 °C and from a few hours to several days (Caligiani, Marseglia, & Palla, 2016; Glicerina & Romani, 2017). However, considering the low  $a_w$  of chocolate and the low temperatures (like 50 °C) used for conching, it is not certain if such a process could serve as a kill step for a 5-log reduction of the target pathogens in chocolate (like *Salmonella*).

The objectives of this research were to 1) study the relationship between moisture content and  $a_w$  at elevated temperature in milk chocolate, 2) determine thermal resistance ( $D$ -value) of *Salmonella* Enteritidis PT 30 in milk chocolate with certain  $a_w$  (0.23, 0.33, and 0.43) at three treatment temperatures (70, 75, and 80 °C).

## 2. Materials and method

### 2.1. Physicochemical properties of milk chocolate

The ingredients (Cocoa liquor 25%, Cocoa butter 25%, Sucrose 20%, Skimmed milk powder 15%, Milk powder 15%) of milk chocolate were purchased online from Amazon. Duplicated samples were sent to Siliker, Inc. (Northern California Laboratory, Salida, CA, USA) for proximate analysis (i.e. ash, carbohydrates, fat, protein) and determination of pH-value. The methods for proximate analysis are described in the Association of Official Analytical Collaboration (AOAC 972.15, AOAC 933.05, AOAC 991.20.1, AOAC 981.12).

### 2.2. Water activity measurement

#### 2.2.1. Sample preparation and conditioning

All ingredients were mixed in 500 mL sterilized beakers, heated in a water bath at 80 °C for 2 h, and re-solidified in a sterile Petri dish (150 mm × 15 mm) by natural cooling (Toker et al., 2019). Solid chocolate bars were ground into powder using a sterile cheddar grater. Before conditioning, chocolate samples were dried in a vacuum oven (ADP-31, Yamato Scientific, Inc., Santa Clara, CA) at 80 °C for 48 h under gauge pressure of 0.08 MPa. The resolidified chocolate was ground into powder using a sanitized cheddar grater. Then the powder was conditioned at  $21 \pm 2$  °C for 3–7 days within five humidity jars filled with different saturated salt solutions ( $\text{CH}_3\text{COOK}$ ,  $\text{MgCl}_2$ ,  $\text{K}_2\text{CO}_3$ ,  $\text{Mg}(\text{NO}_3)_2$ ,  $\text{NaNO}_2$ ) until equilibrium  $a_w$  levels (0.23, 0.33, 0.43, 0.53, and 0.66, respectively) were achieved (Xie et al., 2021). The equilibrated samples were tested using a Water Activity Meter (Model Series 3 TE, Aqualab, METER Group Inc., Pullman, WA) at room temperature ( $21 \pm 2$  °C). The moisture content after conditioning was determined by measuring the weight increase from the conditioning step. All experiments were carried out in triplicate.

#### 2.2.2. Water activity measurement by high temperature cells method (HTC method)

For measurement of water activity change with increasing temperature, 8 g of equilibrated chocolate sample was fitted into each high-temperature cell (HTC) (Washington State University, Pullman, WA,

USA) and closed tightly with a lid. An oil bath (Isotemp 5150 H11, Fisher 180 Scientific, Inc., Pittsburgh, PA, USA) was used to heat the cells to different temperature levels (25, 70, and 80 °C). A polymer-based capacitive humidity sensor (also with temperature function) (Honeywell HumidIcon™ HIH8120, Morristown, NJ, USA) was placed at the center of the lid top to monitor the relative humidity and temperature in the headspace of the cells. The relative humidity and temperature in the headspace above the sample were recorded every minute. At least 10 sustained RH values (10 min) were needed to consider that an equilibrium state was achieved at each temperature. Experimental results were collected from independent triplicated samples.

#### 2.2.3. Data modeling by Clausius-Clapeyron equation

The Clausius-Clapeyron equation (CCE) was used to analyze the experimental data (Tadapaneni, Yang, Carter, & Tang, 2017):

$$a_{w2} = a_{w1} \exp\left(\frac{q_{st}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\right) \quad (1)$$

where  $a_{w1}$  and  $a_{w2}$  are the water activity of the samples at temperatures  $T_1$  and  $T_2$ (K), respectively;  $q_{st}$  is the isosteric heat of sorption (kJ/mol) obtained from the slope of the plotted data ( $\ln a_w$  vs.  $1/T$ );  $R$  is the universal gas constant ( $8.314 \times 10^{-3} \text{ kJ mol}^{-1}\text{K}^{-1}$ ), and

$$q_{st} = a \exp(-b * M_d) \quad (2)$$

where  $M_d$  represents the moisture content (g water/100 g dry solids) of the sample calculated on a dry basis;  $a$  and  $b$  are constants.

### 2.3. Isothermal treatment of *S. Enteritidis* PT30 in milk chocolate

#### 2.3.1. Bacteria strain and inoculation

Because *S. Enteritidis* PT30 is one of the most common strains associated with *Salmonella* outbreaks in low-moisture foods, it was selected as the target pathogen for this research. The original bacteria strain was obtained from the University of California, Davis and stored at  $-80$  °C in tryptic soy broth (TSB, Difco, Sparks, MD, USA) supplemented with 0.6% (w/v) yeast extract (TSBYE) and 20% (v/v) glycerol. Bacteria were activated from frozen stock using two consecutive transfers with TSBYE at 37 °C for 24 h. One mL of the above inoculum was spread on tryptic soy agar supplemented with 0.6% (w/v) yeast extract (TSAYE) plates (150 mm × 15 mm). After 24 h incubation, the bacteria lawn was harvested from the plates with sterile buffered peptone water (BPW). The bacterial solution was centrifuged at  $8000 \times g$ , 4 °C for 10 min. The supernatant was removed, and the pellet was resuspended in 1 mL BPW for further inoculation. The 1 mL inoculum was mixed with 10 g of melted chocolate in a 4 oz Whirl-Pak bag with hand massage for 15 min. Then, added with 90 g melted chocolate sample (melting point,  $\sim 33$  °C) in 16 oz Whirl-Pak bag and also homogenized with hand massage for 15 min until no visible clumps existed. Then the chocolate was naturally solidified and shredded. The chocolate sample was spread onto dishes (150 mm × 15 mm) and conditioned in a humidity chamber for 2–3 days to achieve three different  $a_w$  levels (0.23, 0.33, and 0.43) before isothermal treatment. The  $a_w$  of the conditioned chocolate samples was measured in triplicate before isothermal treatment by a Water Activity Meter (Aqualab, METER Group Inc., Pullman, WA) at room temperature ( $21 \pm 2$  °C).

#### 2.3.2. Isothermal treatment

The isothermal tests for measurement of  $D$ -values of *Salmonella* in chocolate samples were conducted by heating the inoculated samples in thermal death time (TDT) cells (Chung, Birla, & Tang, 2008) using an oil-bath set to constant temperatures. The come-up time (CUT, the time required for sample center temperature to reach within  $0.5$  °C of a specific temperature) was measured with a thermocouple (Type T, OMEGA Engineering, Inc., Stamford, CT, USA) at the geometric center of the sample. The temperature change was recorded using a thermometer

(Digiense DualogR 99100-50, Cole-Parmer Instruments Co., Vernon Hills, IL, USA). The measured temperature of chocolate sample was 69.5 °C, 74.5 °C, and 79.5 °C for the target temperatures (70, 75, and 80 °C). The CUT was from 62 s to 90 s. Each TDT cell was filled with 1 g inoculated and equilibrated chocolate and tightly sealed before isothermal treatments in an oil bath (Isotemp™ 5150 H24, Fisher Scientific™, PA, USA). The TDT cells were removed at five-time intervals after the CUT and quickly submerged in the ice bath for 2 min (Xie et al., 2022). The data was collected in duplicates at each time point. All experimental results were independently conducted in triplicates.

### 2.3.3. Bacteria enumeration

The chocolate sample (1 g) in each TDT cell was scraped using a sterile L-spreader, transferred into 9 mL sterile BPW, and reheated to 37 °C to melt the sample for the convenience of homogenization during serial dilutions. Dilutions were spread onto TSAYE<sup>+</sup> plates (TSAYE supplemented with 0.05% (w/v) ferric ammonium citrate (Sigma-Aldrich, St Louis, MO, USA), and 0.03% (w/v) sodium thiosulfate (Sigma-Aldrich, St Louis, MO, USA)) and incubated at 37 °C for 48 h in two replicates. Colonies that grew with a dark center inside and white outside ring were identified as *Salmonella* cells.

### 2.3.4. Thermal inactivation kinetics

The first-order kinetic model was widely used to describe the survival population of bacteria in low moisture foods (Casulli et al., 2018; Limcharoenchat et al., 2018; Pérez-Reyes, Jie, Zhu, Tang, & Barbosa-Cánovas, 2021; Tsai, Ballom, et al., 2019; Xu et al., 2019). The thermal death curves for *S. Enteritidis* in the tested samples in this study were fit to the first-order kinetic model:

$$\log\left(\frac{N}{N_0}\right) = -\frac{t}{D} \quad (3)$$

Where  $t$  (min) is the treatment time (after CUT) at the target temperature;  $N$  (CFU/g) is the bacterial population at treatment time  $t$ ;  $N_0$  (CFU/g) is the bacterial population after the CUT (90 s);  $D$  (min) is the time to reduce the bacterial population by 90% at the treatment temperature. The  $D$ -value was calculated by the slope of the thermal inactivation curve with log-linear regression.

The  $z_T$ -value (°C) was determined as the temperature increase that results in a 10-fold reduction in  $D$ -value for a sample of a certain  $a_w$ . It was determined from the slope of the regression line that correlated log  $D$ -value and treatment temperature  $T$  (°C) as:

$$\log D_T = -\frac{T}{Z_T} + C \quad (4)$$

where  $C$  is a constant.

Likewise, the  $z_{a_w}$ -value of *S. Enteritidis* is defined as the  $a_w$  increase that results in a 10-fold reduction in  $D$ -value at a certain temperature :

$$\log D_{a_w} = -\frac{a_w}{Z_{a_w}} + C \quad (5)$$

where  $C$  is a constant.

### 2.3.5. Statistical analysis

Data modeling and statistical analysis were conducted using Microsoft Excel (16.51, Microsoft, Redmond, WA, USA). Means and standard deviations (SDs) were calculated for each experimental condition. The goodness of fit was calculated by RMSE (root mean squared error) for all data replicates (Motulsky & Christopoulos, 2004).

$$RMSE = \sqrt{\frac{\sum_{i=1}^n \left[ \log\left(\frac{N}{N_0}\right)_{data, i} - \log\left(\frac{N}{N_0}\right)_{model, i} \right]^2}{n - p}} \quad (6)$$

$\log(N/N_0)$ data,  $i$  is expressed as the log reduction during the thermal

treatment,  $\log(N/N_0)$ model,  $i$  is the predicted log reduction by the model,  $n$  is the number of data replicates, and  $p$  is the degrees of freedom.

## 3. Result and discussion

### 3.1. Proximate analysis

The milk chocolate in this study consisted of fat ( $41.8 \pm 0.6$  g/100 g), carbohydrate ( $40.9 \pm 0.2$  g/100 g), protein ( $13.2 \pm 0.4$  g/100 g), and ash ( $2.8 \pm 0.0$  g/100 g). The moisture content of milk chocolate was  $1.4 \pm 0.0$  g/100 g (wet basis). The pH of milk chocolate was determined as  $6.4 \pm 0.0$ . The raw material of milk chocolate included cocoa butter and sucrose, fat and carbohydrate, made up 82.7% of the total weight of the milk chocolate samples.

### 3.2. The change of $a_w$ and net isosteric heat

The  $a_w$  changes in milk chocolate with increasing temperature at different fixed sample moisture contents are shown in Fig. 1. The sample moisture contents ( $X_w$ ) were 1.2, 1.7, 2.3, 2.8, and 3.9 (dry basis, g/100 g) and the corresponding  $a_w$  were 0.23, 0.33, 0.43, 0.53, and 0.66 at 22 °C. At a given moisture content, the  $a_w$  increased with increasing temperature. For example, the  $a_w$  increased from 0.23, 0.33, 0.43, 0.53, 0.66 at room temperature to 0.34, 0.45, 0.53, 0.58, and 0.70, respectively, at 80 °C. The  $a_w$  increased more with increasing temperature at lower initial  $a_w$  than at the higher  $a_w$ .

The  $a_w$  in low moisture food is highly related to food composition and temperature (Berk, 2008; Syamaladevi et al., 2016). Labuza illustrated that temperature might influence  $a_w$  by affecting the mobility of water molecules and the dynamic equilibrium between the vapor and adsorbed phases (Labuza, 1971). Chocolate consists of carbohydrates, protein, and fat. Hydration of these macromolecules may happen because carbohydrates can dissolve in water by forming hydrogen bonds (Labuza & Altunakar, 2007). The protein is hydrophilic. Hence, the increased temperature is able to break the hydrogen bonds which interact between water and hydrophilic molecules (Iglesias & Chirife, 1977). As a result, a higher temperature is often associated with

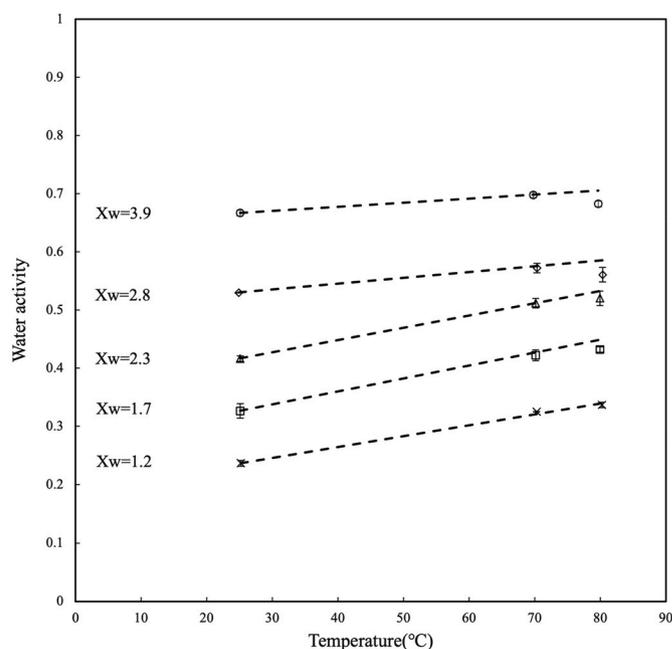


Fig. 1. The relationship between water activity and temperature in milk chocolate.  $X_w$  is the moisture content in milk chocolate (g H<sub>2</sub>O/100 g dry solids). Mean  $\pm$  SD ( $n = 3$ ).

increasing water activity. A similar relationship between temperature and  $a_w$  is also found in high protein (egg powder), high sugar (honey powder, Glucose, Fructose, and other low moisture foods (Alshammari, Xu, Tang, Sablani, & Zhu, 2020; Pérez-Reyes et al., 2021; Xie et al., 2021)). In contrast, the reverse trend can be found in some specific low moisture foods, such as oil, where water activity decreased with increased treatment temperature (Yang, Guan, Sicheng, Sablani, & Tang, 2020). This phenomenon usually happens in peanut butter, oleic acid, and olive oil, which could be attributed to the greater solubility of fatty acids at high temperatures. Consequently, the  $a_w$  of high-fat content foods decreased with a rise in temperature because of the stronger interaction between water and fat molecules (Khuwijitjaru, Adachi, & Matsuno, 2002).

A negative correlation was found between the net isosteric heat ( $q_{st}$ ) and moisture content, i.e.,  $q_{st} = 15.85\exp(-0.742 \cdot X_w)$ , as shown in Fig. 2. The value of  $q_{st}$  was obtained using the Clausius-Clapeyron equation (Eq. (1)), it indicates the amount of energy required for moisture in the samples to change from bound water to vapor (Arslan-Tontul, 2020). According to the prediction equation,  $q_{st}$  was 5.76 kJ/mol at  $X_w = 1.2$ , and it sharply dropped to 0.89 kJ/mol when  $X_w$  reached 3.9. This suggests that the higher moisture content of milk chocolate results in a lower energy requirement for breaking the bond between water and macromolecules.

### 3.3. Thermal resistance of milk chocolate

The initial population of the inoculated *S. Enteritidis* PT 30 in milk chocolate samples was 8.5–8.8  $\log_{10}$  CFU/g. After 3 days of conditioning, the population decreased by about 0.4  $\log_{10}$  CFU/g. The survival curves for *S. Enteritidis* PT 30 in milk chocolate samples treated at three different temperatures are shown in Fig. 3 for 70, 75, and 80 °C, respectively. The goodness of fit for the first order kinetic model was calculated for the thermal resistance test ( $R^2 = 0.90$ – $0.98$ ) at different  $a_w$  and temperature levels, the calculated  $D$ -values are summarized in Table 1. As shown in Table 1, at 80 °C, the  $D$ -values were  $13.6 \pm 1.4$ ,  $9.1 \pm 1.1$ , and  $5.2 \pm 1.0$  min at  $a_w, 80^\circ\text{C} = 0.34$ , 0.45, and 0.53, respectively. That is, it requires 81.6, 54.6, and 31.2 min (6 times  $D$  value) at 80 °C to cause 6-log reduction of *S. Enteritidis* PT 30 in milk chocolate at  $a_w, 80^\circ\text{C} = 0.34$ , 0.45, and 0.53, respectively. At 75 °C,  $D_{75^\circ\text{C}}$ -values decreased to 8.9 min from 30.1 min as the  $a_w$  increased to 0.52 from 0.33. At 70 °C,  $D_{70^\circ\text{C}}$ -values were  $47.4 \pm 3.7$ ,  $31.7 \pm 3.9$ , and  $16.4 \pm 0.4$  min with  $a_w, 70^\circ\text{C} = 0.32$ , 0.43, and 0.51 respectively. In general, higher  $a_w$  resulted in lower  $D$ -values at all treatment temperatures. A similar trend also has been reported for *L. monocytogenes*, *Salmonella*,

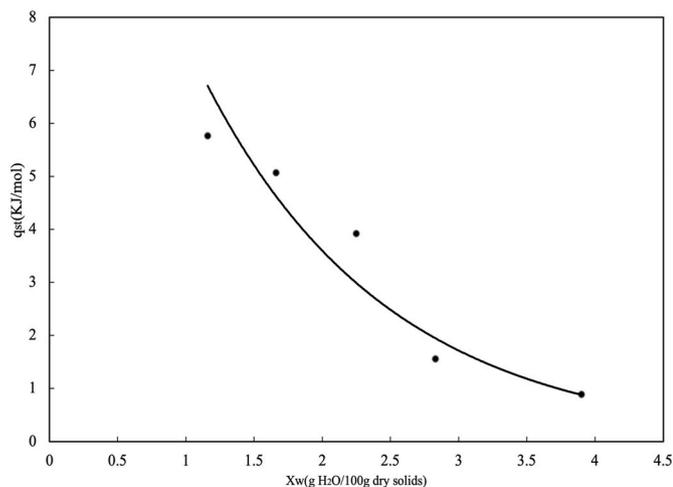


Fig. 2. Net isosteric heat of sorption in milk chocolate. The data was modeled by Clausius-Clapeyron equation (CCE).

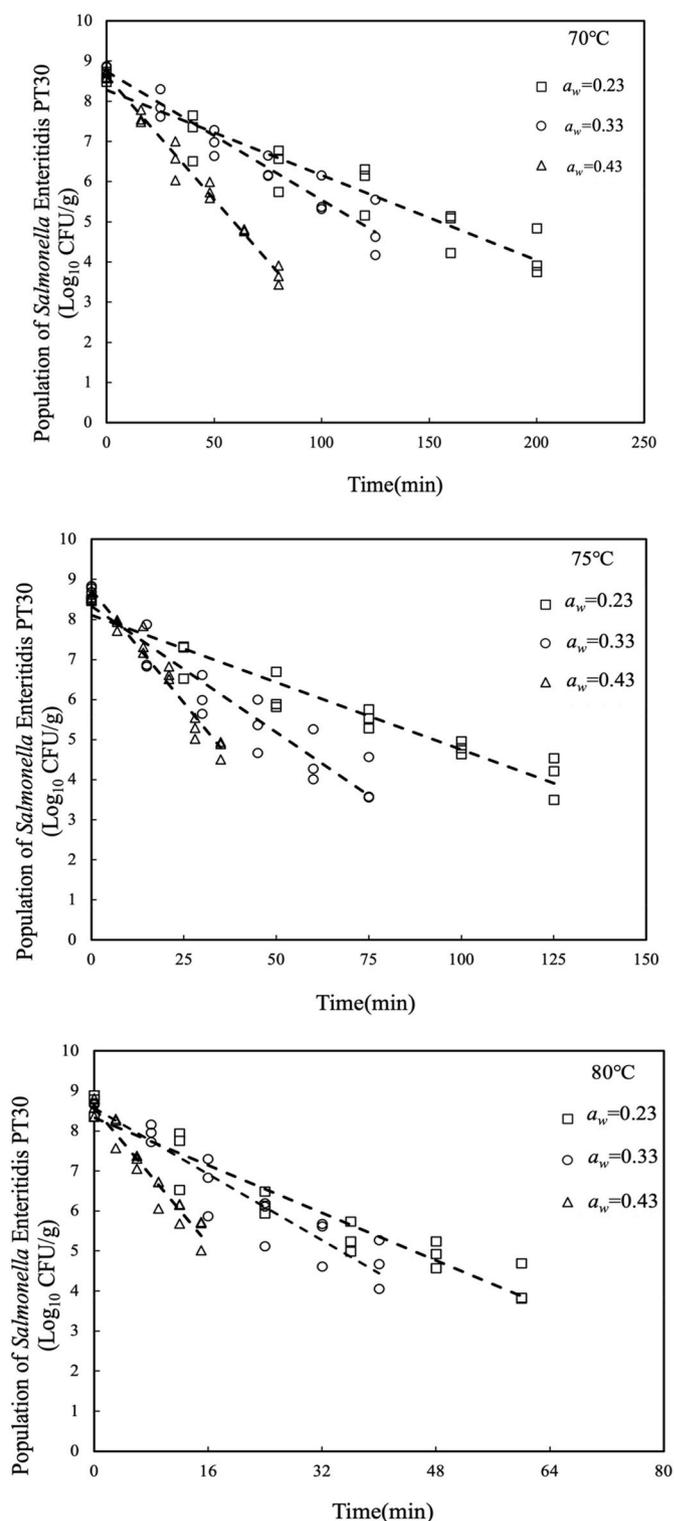


Fig. 3. Thermal inactivation curves of *S. Enteritidis* PT30 in milk chocolate with different equilibrated  $a_w$  (0.23, 0.33, and 0.43) at different temperatures (70 °C, 75 °C, and 80 °C).

and *Enterococcus faecium* in cocoa powder (Tsai, Taylor, et al., 2019).

The thermal resistance of *Salmonella* has been investigated in a wide variety of low moisture foods. It has recently been established that there is an inverse correlation between  $a_w$  at treatment temperature and  $D$ -values of *Salmonella* (Jin, Tang, & Zhu, 2020; Yang, Xu, Lombardo, Ganjyal, & Tang, 2020). For example, recent studies reported  $D$ -value of *S. Enteritidis* PT30 in almonds (fat content 48.8%) and honey powder

**Table 1**

Thermal inactivation data for *S. Enteritidis* PT 30 and log-linear model in milk chocolate (n = 3).

Milk chocolate					
$a_w$ at 25	Temperature (°C)	Treatment $a_w$ (predicted by CCE)	D-value (min)	$z_T$ -value (°C)	RMSE (Log <sub>10</sub> CFU/g)
0.23	70	0.32	47.4 ± 3.7	18.8 ± 2.5	0.49
	75	0.33	30.1 ± 3.0		0.39
	80	0.34	13.6 ± 1.4		0.48
0.33	70	0.43	31.7 ± 3.9	20.6 ± 4.1	0.36
	75	0.44	16.1 ± 1.6		0.54
	80	0.45	9.1 ± 1.1		0.49
0.43	70	0.51	16.4 ± 0.4	18.1 ± 0.5	0.23
	75	0.52	8.9 ± 0.4		0.29
	80	0.53	5.2 ± 1.0		0.29

The data was presented by Mean ± SD (n = 3).

(carbohydrate content 96.4%) to be 21.2 min ( $a_w$ , 80°C = 0.54) and 19.6 min ( $a_w$ , 80°C = 0.50), respectively. In egg white powder (protein content 84.3%), the D-value of *S. PT30* was determined to be 25.8, 13.3, and 6.1 min at  $a_w$ , 80°C = 0.48, 0.56, 0.66, respectively (Pérez-Reyes et al., 2021). Similar correlations were observed for the thermal resistance of *S. Enteritidis* PT30 in milk chocolate and also in other high-fat, high-carbohydrate and high protein content food products. Hence, the observed result supports the theory that  $a_w$  is a key factor to determine the thermal resistance of *Salmonella* in different food products. But the D values for *S. Enteritidis* PT30 in milk chocolate were generally smaller than that in low moisture foods.

Interestingly, unlike most other low-moisture foods, the  $a_w$  of milk chocolate is extremely sensitive to changes in the moisture content  $X_w$  at a given temperature (Fig. 1). This is because chocolate is a high fat content product. The large group of hydrophobics provides less water-binding capacity allowing more free water to compete for the hydrophilic groups in the food matrix. Since the  $a_w$  represents the amount of free water in the food, less  $X_w$  was required to reach the equilibrium  $a_w$  in hydrophobic products than in hydrophilic products (Mermelstein, 2009). This phenomenon usually has been found in high fat content low moisture food such as coconut powder and peanut butter (Jin, Tang, & Sablani, 2019; Syamaladevi et al., 2016). Hence, the moisture content may dramatically affect  $a_w$  and cause the thermal resistance to vary sharply in the chocolate products. This explains the result of Barrile and Cone (1970) who reported that adding extra water into the milk chocolate could decrease the D-value of *S. Anatum* dramatically. That is, a slight change in the moisture content of chocolate may dramatically affect  $a_w$  which in turn would cause the thermal resistance to vary sharply in the chocolate product. Although adding extra water to increase the moisture content is an effective way to weaken the thermal resistance of bacteria, an irreversible separation of fat from the chocolate mass could occur when added water is over 6% (Barrile & Cone, 1970). Moreover, high moisture content alters the rheological property of chocolate and results in sugar bloom (Toker et al., 2019). Sugar absorbs moisture and then dissolves, later forming giant sugar crystals when the moisture is evaporated (Heuler, He, Ambardar, & Voronine, 2020). This phenomenon typically happens with poor quality control during chocolate processing.

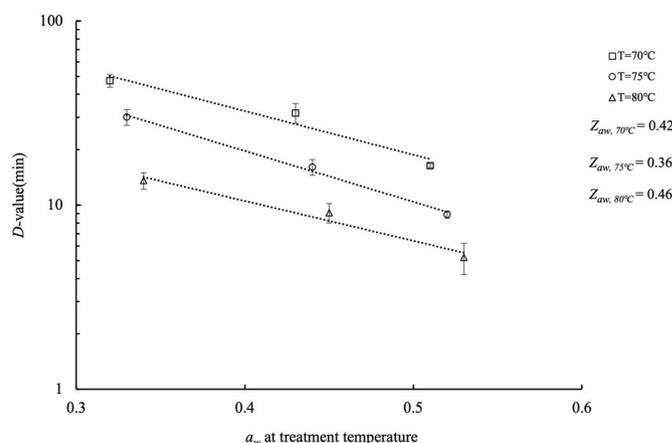
The  $z_T$ -value of *S. Enteritidis* PT 30 in milk chocolate was calculated as 18.8 ± 2.5, 20.6 ± 4.1, and 18.1 ± 0.5 °C at  $a_w$ , room temperature = 0.23, 0.33, and 0.43, respectively (Table 1). A similar study revealed that  $z_T$ -

values of *S. Enteritidis* PT30 in desiccated shredded coconut were 15.9 ± 0.2 and 12.4 ± 0.2 °C at  $a_w$  = 0.25 and 0.45 (Dhowlaghar, Tang, & Zhu, 2021), respectively. 20 °C and 14 °C were observed for  $z_T$ -value of *Salmonella* spp. in cocoa liquor and dark chocolate, respectively (Krapf & Gantenbein-Demarchi, 2010). A wider range of  $z_T$ -values (16.4–26.8 °C) were reported for *Salmonella* spp. in cocoa liquor (Davies, Blood, & Gibbs, 1989), which covers the  $z_T$ -values of 18.8–20.6 °C for *Salmonella* spp. in milk chocolate obtained in this research. The  $z_T$ -values in other chocolate products are close to 20.4 °C (Doyle & Mazzotta, 2000). The similarity of  $z_T$ -values in *Salmonella* illustrates the thermal resistance of bacteria cells that have an similar sensitivity to temperature change in a low moisture environment. However, it has been reported that the  $z_T$ -value decreased to 10.2 °C from 16.9 °C when the moisture content increased to 14% from 10% in wheat flour (Liu, Rojas, Gray, Zhu, & Tang, 2018). The relatively wet environment causes bacteria cells to be more sensitive to temperature changes in thermal treatments.

The  $z_{a_w}$ -values obtained from Eq. (5), illustrate the relationship between  $a_w$  and D-value at a fixed temperature (Fig. 4.). The  $z_{a_w}$ -values of *S. Enteritidis* PT 30 in milk chocolate were 0.46, 0.36 and 0.42 at 80, 75 and 70 °C. This value was higher than previously reported results. For example,  $z_{a_w}$ -values for *Salmonella* PT30 were 0.33, 0.31 and 0.25 at 70, 75 and 80 °C in ground cinnamon, as reported (Xie et al., 2021).  $z_{a_w}$ -value indicates the sensitivity of bacteria thermal resistance, which responds to the environment's  $a_w$  change. The same strain showed  $z_{a_w}$ -value of 0.31 in silicon dioxide at 80 °C (Liu, Tang, Tadapaneni, Yang, & Zhu, 2018). Typically, bacteria can be equilibrated with the surrounding environment by quickly gaining or losing moisture. However, a related study reveals that even though moisture may pass through cocoa due to its porous structure, sugar crystals and coconut butter can effectively block moisture diffusion when compared to cocoa (Ghosh, Duda, Ziegler, & Anantheswaran, 2004). This mechanism supports our results and illustrates the reason why  $z_{a_w}$  of *Salmonella* in milk chocolate is higher compared to other low moisture foods. Alshammari et al. (2020) elaborated a similar mechanism in high sugar products with  $z_{a_w}$ , 80°C -values of 0.91 for *Salmonella* in low moisture fructose powder and 0.80 in honey powder. During thermal treatments, a phase change (from crystal to melt) occurred in fructose and honey powder at  $a_w$ , 80°C = 0.50. The melted sugar may coat the bacterial cells and limit moisture migration from the surrounding environment to enter the bacteria (Alshammari et al., 2020).

#### 4. Conclusion

The  $a_w$  of milk chocolate samples in a closed system increased with increasing temperature at the three tested  $a_w$  levels. The D-value of *S. Enteritidis* PT30 dropped log-linearly with increasing  $a_w$  at the three



**Fig. 4.** The log-scale D-values of *S. Enteritidis* PT 30 with  $a_w$  at different treatment temperatures (70, 75, 80 °C) in milk chocolate. Mean ± SD (n = 3).

treatment temperatures (70, 75, and 80 °C). These results may be useful for the food industry to select appropriate chocolate conching temperature and time for control of *Salmonella* in milk chocolate of a given initial moisture content. It is evident from this study that a small change in moisture content results in a large change in the  $a_w$  of milk chocolate. Thus, special care should be taken to prevent moisture loss during conching, as a drop in moisture content reduces water activity which in turn sharply increases bacterial thermal resistance. Future research should focus on the thermal inactivation of multiple bacterial strains in different chocolate products (i.e., cocoa liquor, chocolate syrup). The effect of different chocolate recipes on bacterial thermal resistance should be investigated because of the complicated structures and diverse components found in chocolate products.

#### CRedit authorship contribution statement

**Sicheng Sun:** Conceptualization, Methodology, Investigation, Writing – original draft. **Yucen Xie:** Investigation, Methodology, Writing – review & editing. **Ren Yang:** Investigation, Methodology, Writing – review & editing. **Mei-Jun Zhu:** Writing – review & editing. **Shyam Sablani:** Writing – review & editing. **Juming Tang:** Supervision, Funding acquisition, Writing – review & editing.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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