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Survival and thermal resistance of *Salmonella* in chocolate products with different water activities

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ABSTRACT

Contamination of *Salmonell*a in chocolate products has caused worldwide outbreaks and recalls. There is a lack of information on the impact of water activity (a_w) on the stability of *Salmonella* in chocolate products during storage and thermal treatments. In this research, the survival and thermal resistance of a *Salmonella* cocktail (*S.* Enteritidis PT30, *S.* Tennessee K4643, *S.* Typhimurium S544) was examined in different chocolate products (dark chocolate, white chocolate, milk chocolate) at two a_w levels (0.25, 0.50) over 12 months at 22 °C. A reduction of 4.19 $\log_{10}$ CFU/gof *Salmonella* was obtained in dark chocolate after 12 months (a_w = 0.50, at 22 °C); less reductions were observed in white and milk chocolates. In all three products, more reductions were observed ata_w = 0.50 than at a_w = 0.25 over the 12-months storage. When treated at 80 °C, the *D*-values (time required to cause 1 log reduction) of the *Salmonella* cocktail in the chocolate samples with initial a_w of 0.25 were 35.7, 25.2 and 11.6 min in dark, white and milk chocolate, respectively, before the storage. The $D_{80°C}$ -values of *Salmonella* cocktail in the samples with initial a_w of 0.50 were 6.45, 7.46, and 3.98 min in dark, white and milk chocolate, respectively. After 12 months of storage at 22 °C, the $D_{80°C}$-value of *Salmonella* cocktail decreased to 9.43 min ($p < 0.05$) in milk chocolate but remained 22.7 min in white chocolate with an a_w of 0.25 at 22 °C. The data suggests that *Salmonella* can survive in chocolate products for up to 12 months, and its thermal resistance remained relatively stable. Thus, *Salmonella* is resistant to desiccation in chocolates, particularly in milk and white chocolates, and its thermal resistance remains during one-year storage, which could pose a potential threat for future outbreaks.

1. Introduction

Salmonella is a foodborne pathogen known to cause severe illness in susceptible populations. It is one of the primary bacteria responsible for foodborne illness in the United States (Wittler, 2023). The severity of the threat posed by *Salmonella* is due to its remarkable ability to withstand harsh environmental conditions, such as temperature, pH, desiccation (Podolak et al., 2010). *Salmonella* can survive for extended periods in low-moisture foods or environments (Abdelhamid & Yousef, 2020). *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) is the predominant strain of *Salmonella* and a primary contributor to foodborne illnesses in the United States (Tack et al., 2020). A recent outbreak of *Salmonella* Typhimurium has impacted chocolate products, resulting in 369 reported infections across European Union countries (Samarasekera, 2022). In a previous *Salmonella* outbreak, 439 cases of infection were reported in Europe after the consumption of contaminated chocolate products (Werber et al., 2005). These incidents highlight the persistent challenges faced by food manufacturers in their efforts to mitigate the risk of *Salmonella* throughout the chocolate supply chain.

Chocolate is a low-moisture food with a moisture content below 2% and is known for its high sugar and fat contents (Krapf & Gantenbein-Demarchi, 2010). The main ingredient of chocolate products is coco beans, which are harvested from *Theobroma cacao* (Afoakwa, Paterson, & Fowler, 2007; Dezena, 2021). During the processing of chocolate, cocoa beans are mixed with other ingredients such as cocoa butter, sugar, and emulsifier (Stortz & Marangoni, 2011). Water activity (a_w) is a measurement of the availability of water for biological reactions. Different microbial groups have varying requirements for growth, with some microbial groups are capable of growth at an a_w as low as 0.60, while others require much higher levels (Dilbaghi & Sharma, 2007). Water activities of chocolate products in storage and markets ranged from 0.3 to 0.5, which can vary depending on processing conditions and

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compositions (Krapf & Gantenbein-Demarchi, 2010). *Salmonella* can survive at low a_w foods for an extended period (Podolak et al., 2010). For example, only 1.17 log CFU/g reduction of *Salmonella* in vacuum-packed halva (a_w = 0.18) occurred during eight months storage at 18–20 °C (Kotzekidou, 1998). Flock et al., (2022) reported that *Salmonella* remained above 6.0 $\log_{10}$ CFU/g (7.0–9.0 $\log_{10}$ CFU/g inoculated populations) in boiled eggs (a_w = 0.11) and chocolate protein drink powder (a_w = 0.37) after 12 months of storage at 25 °C. In this regard, there have been no published reports about the influence of a_w on survival of *Salmonella* in chocolate products during storage and on the changes in the thermal resistance of *Salmonella* after storage.

Numerous studies have confirmed that a low a_w environment contributes to increased thermal resistance of *Salmonella* in food products (Xie et al., 2021a; Xie et al., 2021b; Sun et al., 2022; Tsai et al., 2019; Zhu et al., 2021).The low a_w environment causes dehydration of bacterial cells, leading to an enhanced resistance to heat damage (Xie et al., 2021a; Cebrián et al., 2017). Furthermore, Unfermented cocoa beans are rich in polyphenols (12–18%, dry weight) before processing (Jalil & Ismail, 2008). Previous studies reported that cocoa extract had antimicrobial activities against *Escherichia coli* and *Salmonella* (Nsor-Atindana et al., 2012; Budaraga & Putra 2019). Tetramethylpyrazine (TMP), an antibacterial agent, was found in in fermented cocoa beans and vinegar (Cherniienko, Pawełczyk, & Zaprutko, 2022; Xu et al., 2022). Liu et al. (2019) reported that TMP could reduce the *S.* Typhimurium load in broilers. To develop effective thermal treatments, it is essential to establish quantitative relationships that link the thermal resistance of *Salmonella* to chocolate products.

Conching is an important thermal treatment in chocolate production to achieve a homogeneous blend of chocolate ingredients. The specific conching conditions in the chocolate industry vary depending on the type of the chocolate product being produced. Typically, the conching temperature falls within the range of 50–80 °C, while the duration of conching process spans from a few hours to several days (Wollgast & Anklam, 2000; Afoakwa, 2016). This process ensures desired flavor, texture, and overall quality of the final chocolate product (Toker, Palabiyik, & Konar, 2019 ; Krapf & Gantenbein-Demarchi, 2010). In this research, we selected the range of temperature and time relevant to industrial counching operations to examine the survival and thermal resistance of *Salmonella* in various chocolate products.

The objectives of this research were to:1) investigate the survival of *Salmonella* in dark, white and milk chocolates during 12 months of storage at 22 °C at two a_w(0.25 and 0.5); 2) determinechange in thermal resistance of *Salmonella* cocktail in chocolate products at 80 °C after -12months storage.

2. Materials and methods

2.1. Sample preparation

Ingredients were purchased from a local chocolate store to prepare dark, milk and white chocolate based on published recipes (Tamminga et al., 1976; Vercet 2003; Jasson et al., 2011). The ingredients were mixed in 500 mL sterilized beakers, melted in a water bath at 80 °C for 2 h, and re-solidified on a sterile petri dish (150 mm × 15 mm) at 22 °C according to a modified protocol from Toker, Palabiyik, & Konar (2019).

2.2. Physicochemical properties of chocolate products

The proximate compositions (ash, carbohydrates, fat, and protein) of the products were determined by Silliker, Inc. (Northern California Laboratory, Salida, CA, USA) in duplicate using standard analytical methods (Latimer, 2012). The methods of the proximate analyses followed those of the Association of Official Analytical Collaboration (AOAC 972.15, AOAC 933.05, AOAC 991.20.1, AOAC 981.12).

2.3. Bacterial strains

S. Enteritidis PT30, *S.* Tennessee K4643, and *S.* Typhimurium S544 were used to prepare a *Salmonella* cocktail for this study. These strains were selected based on their association with recorded outbreaks in low moisture food (D'aoust, 1977; FSN, 2022). The bacterial strains (*S.* Enteritidis PT30 was obtained from the University of California, Davis. *S.* Tennessee K4643 and *S.* Typhimurium S544 were obtained from FDA, Chicago, Illinois) were maintained at −80 °C in tryptic soy broth (TSB, Difco™, Detroit, MI, USA), supplemented with 0.6% (w/v) yeast extract (Fisher Scientific, Pittsburgh, PA, USA) (TSBYE) and 20% (v/v) glycerol.

2.4. Culture preparation and bacterial inoculation

The thawed culture stocks were subjected to two consecutive transfers in 9 mL of TSBYE for 24 h at 37 °C. 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) (Hildebrandt et al., 2016). The bacteria were collected from plates with 40 mL sterile buffered peptone water (BPW) using a sterile L-spreader. After lawn harvest from the plates, the bacterial suspension was centrifuged at 8000 × g, 4 °C for 10 min. The populations of the *Salmonella* cocktail inoculum were assessed by plating suitable dilutions on TSAYE agar, followed by incubation at 37 °C for 48 h. After incubation, the bacterial colonies were counted to determine the population size. An equal volume (1 mL) of each *Salmonella* serotype was combined in a conical tube and mixed together to reach a population of 10 ~ 11 $\log_{10}$ CFU/mL for inoculation. Before inoculation, an examination of the microflora present in uninoculated chocolate samples were conducted to confirm the absence of microbial contamination in the raw chocolate material. To prevent cross-contamination, all the tools used were autoclaved. The solid chocolate bar was preheated to create a chocolate paste at 35 °C in an oven. One mL of inoculum was added to 10 g of preheated chocolate paste in a 4 oz Whirl-Pak bag (Nasco™, Fisher Scientific) and mixed by hand massage for 5 min to obtain homogeneity. Then, 90 g of chocolate paste was added to 10 g inoculated chocolate in a 16 oz Whirl-Pak bag and mixed with a repeat of the hand massage for 15 min. The uniformity of the inoculated sample was assessed by randomly taking samples from 5 different locations for enumeration of *Salmonella*. Next, the 100 g of inoculated chocolate paste in the bag was covered with aluminum foil and left in the hood overnight to allow for solidification. The following morning the bar samples were shredded using a sterile grater. A well-sealed humidity chamber was used to condition shredded chocolate to different a_w levels (0.25 and 0.50) at 22 °C for 2–3 days. The a_w of conditioned chocolate samples was measured using a Water activity meter (Aqualab, METER Group Inc., Pullman, WA) at 22 °C.

2.5. Salmonella survival in chocolate products

The above prepared chocolate samples were divided into smaller portions and placed in 4 oz Whirl-Pak bags (analytical sample, ~5 g each). To avoid water loss during storage, these sample bags were sealed in moisture barrier bags (Dri-Shield 3000®, Desco Industries, Inc) using an impulse sealer (model FS-400), and then subjected to 12 months of storage at 22 °C. The moisture barrier bags were manufactured from nylon, foil, and polyethylene (water vapor transmission rate < 0.0003 g/100 m^2. day). The initial populations of the *Salmonella* cocktail in the inoculated chocolate samples at a_w = 0.25 ranged between 8.6 $\log_{10}$ CFU/g and 8.8 $\log_{10}$ CFU/g. In the chocolate samples with an a_w of 0.50, the initial populations of *Salmonella* cocktail were 8.1–8.2 $\log_{10}$ CFU/g. These initial populations were considered as the day 0 data for the storage studies. The chocolate samples under storage were tested weekly or monthly over a 12-month period. Experiments were conducted independently in duplicate; for each sampling point, three samples were analyzed.

2.6. Thermal resistance of Salmonella in chocolate products

To determine the thermal resistance of the *Salmonella* cocktail in chocolate samples prepared at the two a_w levels, thermal treatments of the inoculated samples were performed using the Thermal-Death-Time test cells (TDT cell II, Fig. 1.) and a glycol oil bath (Isotemp™ 5150 H24, Fisher Scientific™, PA, USA) with a circulator (Chung, Birla, & Tang, 2008). Compared to TDT cell I (Fig. 1.) which was used in previous research (Sun et al., 2022), TDT cell II offers the advantage of shorter come-up time (CUT) during the thermal treatment. The CUT refers to the time required for the temperature at the geometric center of the sample to reach within 0.5 °C of a specific temperature. The CUT time for three chocolate products ranged from 50 s to 1 min 5 s. The chocolate samples (pre-conditioned to $a_w = 0.25$ or 0.50) were sealed in TDT cell II (1.0 g per each cell), and then immersed in an oil bath at 80 °C. The TDT cells were removed from the oil bath at five specific time points following the CUT time and quickly submerged in an ice bath for 2 min to stop inactivation (Sun et al., 2022). The thermal treatments were performed before and after 12-month storage. The experiments were repeated in duplicate, and for each sampling point, two samples were analyzed.

2.7. Enumeration of Salmonella

The thermally treated chocolate sample was scraped from the TDT cell II (after submerged in an ice bath for 2 min) using a sterile L-spreader. For storage study, the chocolate samples were taken from well-sealed moisture barrier bag at 22 °C. One gram chocolate sample was transferred into 9 mL sterile BPW (Liu et al., 2018) in a glass tube, preheated to 35 °C to facilitate homogenization, and vortexed for 30 s. The resulting homogenate was subjected to 10-fold serial dilutions. Appropriate dilutions were then spread onto TSAYE (general-purpose media) plates supplemented with 0.05% (w/v) ferric ammonium citrate (Sigma-Aldrich, St Louis, MO, USA). The inoculated plates were then incubated at 37 °C for 48 h. The colonies that displayed a dark center inside and a white outside ring were identified as *Salmonella* cells (Liu et al., 2022).

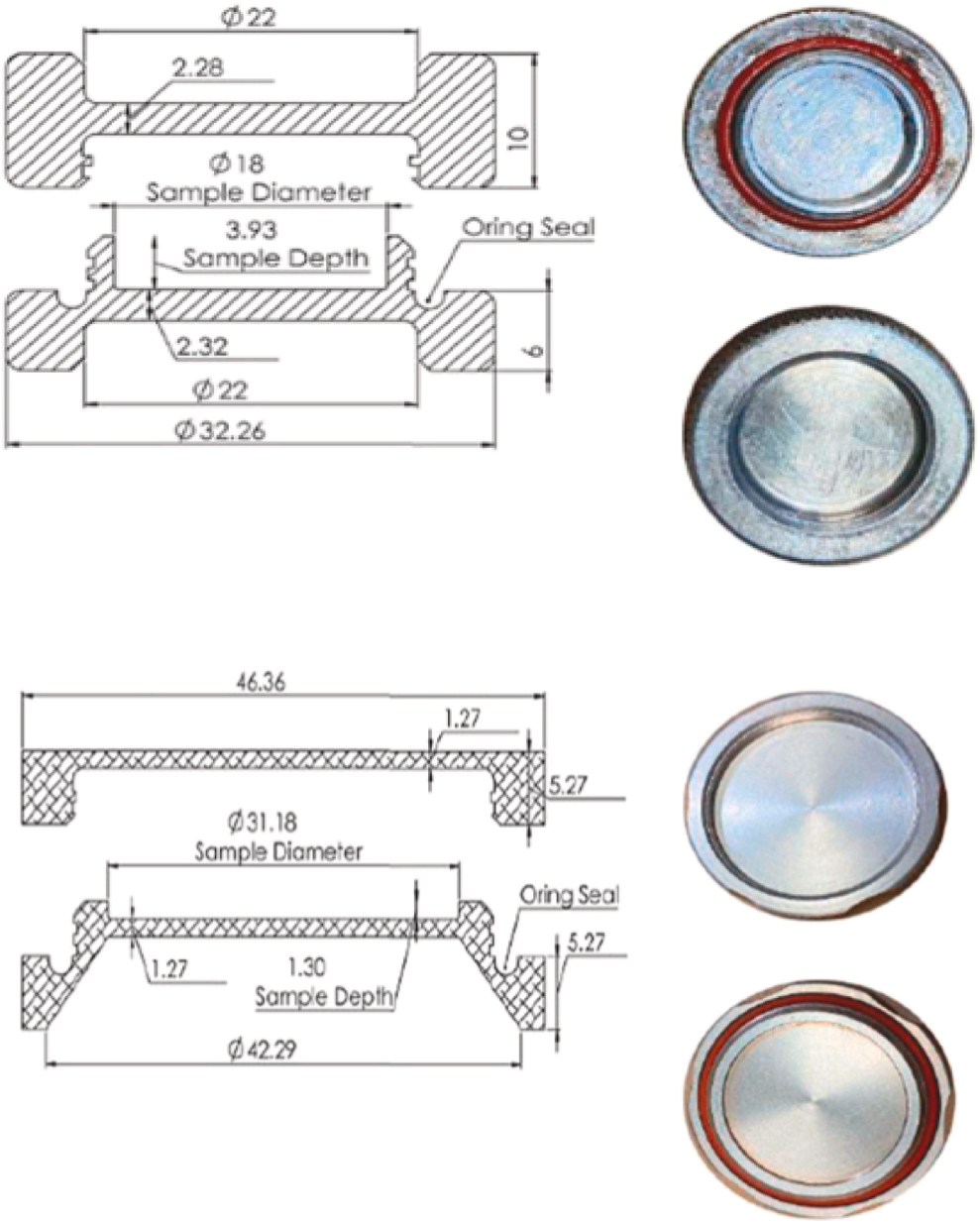

Fig. 1. Schematic and physical diagrams (the dimensions are in mm) of TDT cell I (top) and TDT cell II (bottom) for low moisture foods (Cheng et al., 2021; Jin & Tang, 2019).

2.8. Thermal inactivation kinetics

The first-order kinetic model was used to describe the inactivation of *Salmonella* cocktail in this study which can be expressed as below (Peleg, 2006):

$$\log(\frac{N}{N_0}) = -\frac{t}{D} \quad (1)$$

where t (min) is the treatment time (after CUT, i.e., after reaching within 0.5°C of the treatment temperature); N (CFU/g) is the bacterial population at treatment time t; N_0 (CFU/g) is the bacterial population after the CUT (~60 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 fitness of the model was quantified using the coefficient of determination (R^2) and Root Mean Square Error (RMSE).

2.9. Statistical analysis

Data modeling and statistical analyses were done using Microsoft Excel (16.51, Microsoft, Redmond, WA, USA). Means and standard deviations (SDs) were calculated using RStudio (Boston, MA, USA) for each experimental condition. Tukey multiple comparison test was used for two-way ANOVA.

3. Results and discussion

3.1. Proximate analysis

Table 1 shows the chemical compositions of three chocolate products. Milk chocolate had the highest carbohydrate content at 40.9%, while dark and white chocolate had 15.2% and 30.1%, respectively. The fat content was 74.3% in dark chocolate, 53.0% in white chocolate, and 41.8% in milk chocolate. Milk chocolate and white chocolate also had a higher protein content of 13.2% and 12.4%, respectively, compared to 8.17% for dark chocolate. As expected, chocolate products are known for their high levels of carbohydrates and fat, owing to their primary ingredients are sugar and cocoa butter. Although proximate analysis does not provide a direct assessment of antimicrobial ingredients, it serves as an essential initial step in understanding the chemical composition of chocolate products, which, in turn, affect the survival and thermal resistance of *Salmonella* (Xu et al., 2019).

3.2. Survival of Salmonella in chocolate products at 22 °C during storage

The survivals of *Salmonella* in three chocolate products with a_w of 0.25 or 0.50 over the 28 days at 22 °C are shown in Fig. 2. The initial concentrations of *Salmonella* in dark, milk, and white chocolate products were 8.6–8.8 $\log_{10}$ CFU/g for the chocolate products with a_w of 0.25, and between 8.1 and 8.2 $\log_{10}$ CFU/g for the chocolate products with a_w of 0.50. The populations of *Salmonella* slightly decreased with storage time. After 28 days of storage, a 0.60, 0.83 and 0.89 $\log_{10}$ CFU/g reduction was observed in the milk, dark, and white chocolate samples at $a_w = 0.25$. A reduction of 0.86, 0.97, and 1.20 $\log_{10}$ CFU/g were obtained in milk, dark, and white chocolate samples at $a_w = 0.50$. The reduction of *Salmonella* in white chocolate at $a_w = 0.50$ was higher ($p < 0.05$) compared to that at $a_w = 0.25$ over 28 days of storage at 22 °C. However, the population of *Salmonella* remained relatively stable in dark and white chocolate after 28 days of storage at 22 °C.

During the 12-month storage at 22 °C, a reduction of 3.83 $\log_{10}$ CFU/g (Fig. 3.) was observed in dark chocolate with an a_w of 0.25. This reduction was higher ($p < 0.05$) than that in white and milk chocolate (2.17 $\log_{10}$ CFU/g and 2.98 $\log_{10}$ CFU/g at $a_w = 0.25$). More reductions were observed in the chocolate products with an a_w of 0.50. The population of *Salmonella* cocktail in dark chocolate was reduced by 4.19 $\log_{10}$ CFU/g, while reductions of 2.52 and 3.13 $\log_{10}$ CFU/g were observed in white and milk chocolate, respectively. This finding aligns with a study on almond meal, where *Salmonella* decreased by 1.5 $\log_{10}$ CFU/g at an a_w of 0.45 and 0.8 $\log_{10}$ CFU/g at an a_w of 0.25 after 52 weeks of storage at 22 °C (Zhu et al., 2021). Similarly, Lian et al., (2015) reported reductions of 4.2, 4.7, and 5.9 $\log_{10}$ CFU/g of *Salmonella* in skim milk powder at $a_w = 0.33$, 0.53, and 0.81, respectively, after two months of storage. Dormancy plays a crucial role in the survival of *Salmonella*. When exposed to stressful environmental changes, *Salmonella* enters the viable but nonculturable (VBNC) state, where it remains alive but unable to develop in that particular environment (Salive et al., 2020). Once conditions become favorable again, these cells transition back to a normal metabolic state (Salive et al., 2020).

Food composition plays a crucial role in the survival of *Salmonella*. Chocolate is a high-sugar and fatty food product. He et al., (2011) reported a greater *Salmonella* reduction in natural peanut butter (50% fat, 22% carbohydrate) than in reduced-fat peanut butter (33% fat, 42% carbohydrate). However, our results showed that *Salmonella* survived better in milk and white chocolate (41.8% and 53.0% fat, Table 1) than dark chocolate (74.3% fat, Table 1) during 12 months of storage, regardless of a_w. This might have been caused by antimicrobial compounds in cocoa beans. TMP's antimicrobial effect has also been studied in relation to the activation of epithelial antimicrobial peptides (Ding et al., 2019). In addition, the presence of polyphenol in cocoa beans may also contribute to antimicrobial activities in chocolates. For example, the cocoa extract incorporated into food packaging film exhibited antimicrobial activities against *Salmonella* in a commercial infant milk formula (Calatayud et al., 2013). According to recipes of different chocolate products, dark chocolate contains a higher level of cocoa content than milk and white chocolates (55% in dark chocolate vs. 25% in milk chocolate and 0% in white chocolate). Hence, our results suggest that the higher content of cocoa beans in dark chocolate might contribute to a more substantial decrease in *Salmonella* population. The antimicrobial compounds present in cocoa beans may exert an influence on the survival of *Salmonella* in different chocolate products, particularly when subjected to long-term storage at different a_w levels. However, it is important to note that despite the presence of antimicrobial compounds in chocolates, *Salmonella* managed to remainviable during 12-month room temperature storage.

3.3. Thermal resistance of Salmonella in chocolate products during storage

Before storage, the $D_{80°C}$-values of the *Salmonella* in dark, white, and milk chocolate with initial a_w of 0.25 were 35.7 ± 3.3, 25.2 ± 2.7, and 11.6 ± 0.5 min, respectively. After 12 months of storage, the $D_{80°C}$-values of *Salmonella* in the white and milk chocolate samples with initial a_w of 0.25 decreased to22.7 ± 1.1 min and 9.43 ± 1.01min, respectively (Table 2). Before storage, $D_{80°C}$-values of *Salmonella* in dark, white, and milk chocolate at $a_w = 0.50$ were 6.45 ± 0.75, 7.46 ± 0.80, and 3.98 ± 0.20 min, respectively (Table 2). We were not able to accurately measure $D_{80°C}$-values of the *Salmonella* cocktail in the dark chocolate samples at a_w of 0.25 and the samples at a_w of 0.5 after 12 months storage because of the lowpopulations (<6.0 $\log_{10}$ CFU/g) (Table 2).

The thermal death curves are presented in Fig. 4. Before storage, The $D_{80°C}$-value of *Salmonella* in the chocolate samples was generally lower

Table 1
Proximate analysis of chocolate products.

	Milk Chocolate	Dark Chocolate	White Chocolate
Ash (%, w/w)	2.75 ± 0.01	1.70 ± 0.02	2.61 ± 0.01
Carbohydrate (%, w/w)	40.9 ± 0.2	15.2 ± 0.1	30.1 ± 0.2
Fat (%, w/w)	41.8 ± 0.6	74.3 ± 0.1	53.0 ± 0.2
Moisture (%, w/w)	1.44 ± 0.03	0.63 ± 0.02	1.83 ± 0.11
Protein (%, w/w)	13.2 ± 0.4	8.17 ± 0.20	12.4 ± 0.0

Mean ± SD, n = 2.

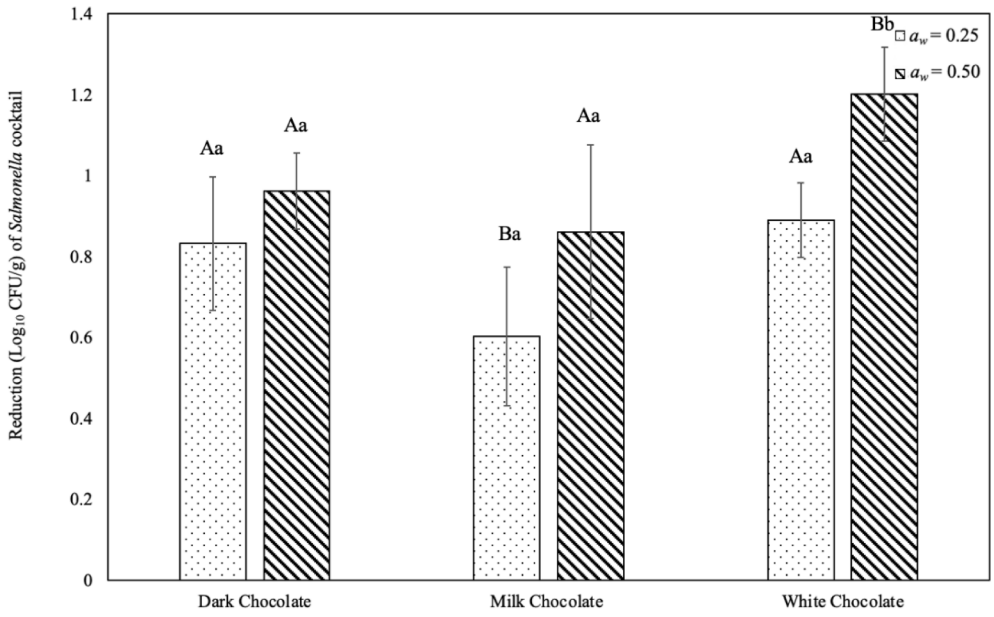

Fig. 2. The reduction of *Salmonella* in three chocolate products with a_w 0.25 or 0.50 after 28 days of storage at 22 °C. Lowercase letters represent significant differences in the reduction of *Salmonella* in the same type of chocolate at different a_w levels. Uppercase letters represent significant differences in the reduction of *Salmonella* among different types of chocolate at the same a_w level. Mean ± SD, n = 3.

at a higher a_w level ($a_w = 0.50$). a_w is a factor that can strongly affect *D*-value of bacteria. As long as an a_w gradient is formed between the bacteria cell and food, the moisture diffusion drives the bacteria to gain or lose the water until the system reaches equilibrium (Xie et al., 2021a). Heating under a high moisture condition can induce irreversible destabilization of ribosomes in bacteria cells (Mackey et al., 1991). However, desiccation may stabilize ribosomes and reduce molecular mobility, thereby mitigating heat damage (Syamaladevi, 2016).

After 12-month storage, the $D_{80°C}$-value of *Salmonella* in milk chocolate significantly decreased ($p < 0.05$). But the 12-month storage did not change $D_{80°C}$-value of *Salmonella* in white chocolate ($p > 0.05$). Previous studies have reported stability in thermal resistance of bacteria during storage, such as *L. monocytogenes* in chicken meat powder, which showed no significant change in the $D_{80°C}$-value of *Salmonella* after 21 days at 16 °C (Rachon, Peñaloza, & Gibbs, 2016). Additionally, *Salmonella* was reported to maintain the similar thermal resistance after 12 months of storage at different temperature in almond meal with different a_w levels (Zhu et al., 2021). However, due to the influence of antimicrobial compounds, the thermal resistance of *Salmonella* could be affected during storage. Polyphenol rich in cocoa beans may also contribute to antimicrobial activities in chocolates. The cocoa extract containing food packaging film shows antimicrobial activities against *E. coli* and *Salmonella* (Calatayud et al., 2013; Nsor-Atindana et al., 2012; Budaraga & Putra 2019). In support of our findings, Xie et al., (2022) reported that the $D_{70°C}$-values of *Salmonella* in chili, cinnamon, and black pepper decreased to 7.7, 8.8, and 28.9 min from 15.4, 20.8, and 36.6 min, respectively, after one-year storage. The comparison of the $D_{80°C}$-value of *Salmonella* in milk and white chocolate during storage suggests that antimicrobial compounds in milk chocolate samples might have altered the thermal resistance of bacteria.

It is interesting to note that the highest $D_{80°C}$-value of *Salmonella* was observed in dark chocolate samples which contained the highest fat content (74.3%) compared to white (53.0%) and milk chocolate (41.8%) at $a_w = 0.25$. Biehl and Ziegleder (2003) stated that cocoa butter could protect bacterial cells and allow them to resist the gastric acid in the stomach. Without cocoa butter, cocoa powder also caused a much lower $D_{80°C}$-value of *Salmonella* (11.5–7.0 min, $a_{w,\ 22°C} = 0.30–0.45$) (Tsai, et al., 2019) compared to the $D_{80°C}$-value obtained in dark chocolate in our study. Several studies have reported that a high-fat content and a dry environment exert a protective effect on pathogens exposed to heat damage (Kim et al., 2017; Li et al., 2018; Xie et al., 2021a). This protective effect is believed to be associated with minimal changes in a_w at elevated temperatures, especially when compared to products containing high levels of carbohydrates or proteins (He et al., 2011; Yang et al., 2020). The disparity in a_w between bacterial cells and the food generates a moisture vapor pressure gradient, causing moisture to diffuse in or out of the bacterial cells until equilibrium is achieved within seconds (Xie et al., 2021a). When the food system provides a lower a_w, the bacterial cells experience dehydration. This dehydration can impact protein aggregation and the functionality of DNA and RNA, which are key cellular targets for heat-induced microbial inactivation (Cebrián et al., 2017). The loss of moisture from microbial cells during thermal treatment thereby increasing the thermal resistance of bacteria cells to heat damage (Syamaladevi et al., 2016).

4. Conclusion

The populations of *Salmonella* in the inoculated chocolate products decreased with storage time. 2.0 – 4.2 $\log_{10}$ reductions of *Salmonella* were observed over the 12-months storage. Greater reductions of bacteria were associated with higher water activity. The survival of *Salmonella* followed the order of white chocolate > milk chocolate > dark chocolate. That is, the dark chocolate, with its higher cocoa content, exhibited a more potent antimicrobial effect compared to white or milk chocolate in storage. Moreover, the thermal resistance of *Salmonella* decreased in milk chocolate, but remained stable in white chocolate after 12-months storage. The information obtained from this study provide valuable insights that can assist manufacturers in designing appropriate storage conditions and improving microbial safety in different chocolate products. Future studies should investigate the changes in a_w at elevated temperatures in various chocolate products, given that a_w is a temperature-dependent parameter influenced by product composition. Understanding how a_w of specific products changes in thermal treatments should allow more precise determination of the relationship between a_w and the thermal resistance of *Salmonella* within the food matrix.

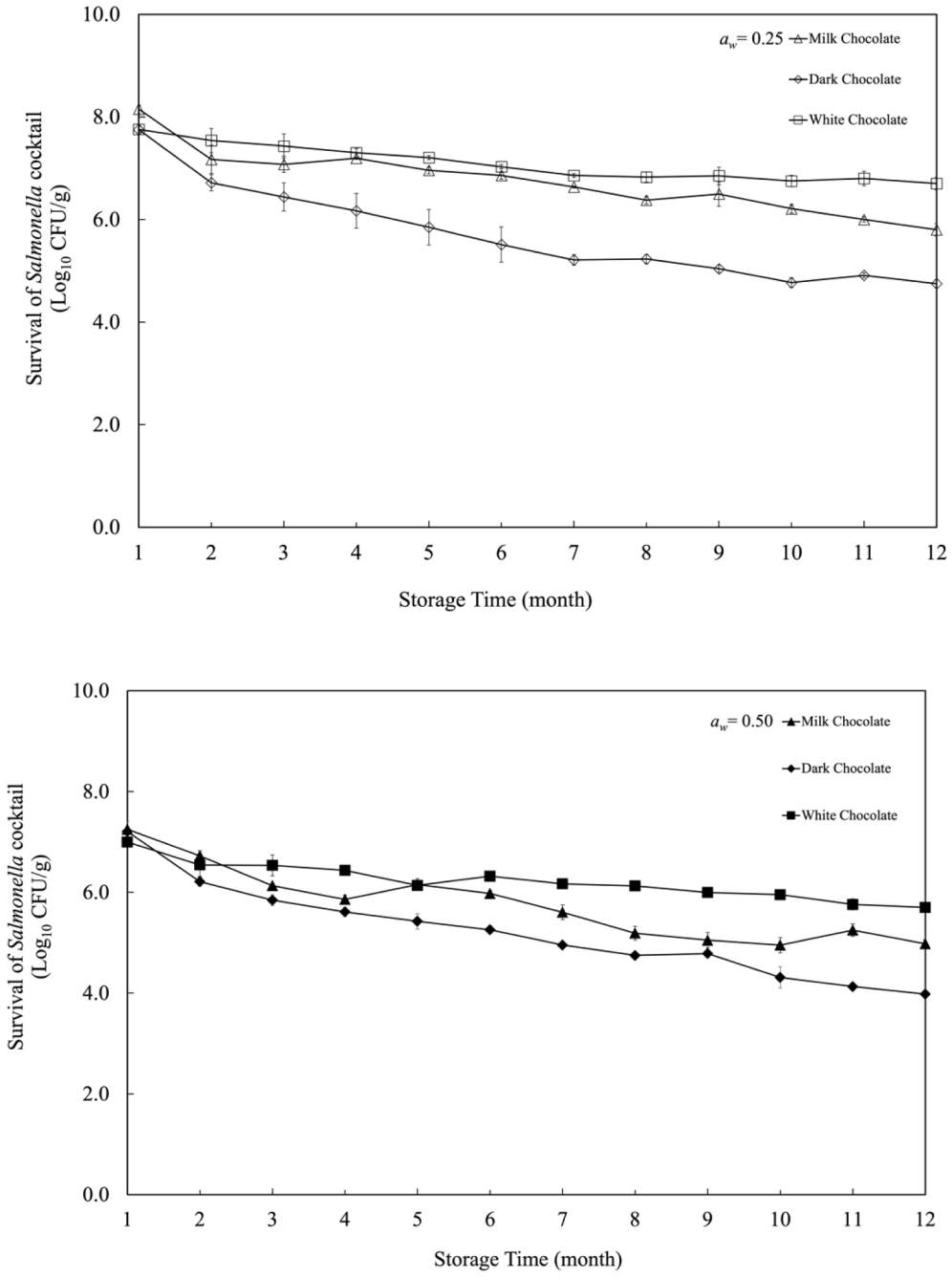

Fig. 3. The survival of *Salmonella* in three chocolate products with different a_w levels (0.25, 0.50) over the 12 months of storage at 22 °C. Reduction rate of *Salmonella* in Milk, Dark and White chocolate were 0.25, 0.32, 0.18 log_{10} CFU/g per month for $a_w = 0.25$ and 0.26, 0.35, 0.21 log_{10} CFU/g per month for $a_w = 0.50$. Mean ± SD, n = 3.

Table 2
The $D_{80°C}$-values of *Salmonella* cocktail before and after 12-month storage with different a_w levels at 22 °C.

Chocolate products	a_w at 22 °C	$D_{80°C}$-value(min)					
		month 0	R^2	RMSE (Log_{10} CFU/g)	month 12	R^2	RMSE (Log_{10} CFU/g)
Dark Chocolate	0.25	35.7 ± 3.3^{A}	0.97	0.40	–	–	
White Chocolate	0.25	25.2 ± 2.7^{Ca}	0.96	0.30	22.7 ± 1.1^{Ba}	0.92	0.35
Milk Chocolate	0.25	11.6 ± 0.5^{Ba}	0.96	0.31	9.43 ± 1.01^{Ab}	0.91	0.23
Dark Chocolate	0.50	6.45 ± 0.75^{A}	0.90	0.46	–	–	
White Chocolate	0.50	7.46 ± 0.80^{A}	0.96	0.30	–	–	
Milk Chocolate	0.50	3.98 ± 0.20^{B}	0.95	0.32	–	–	

Lowercase letter shows the significant difference between the $D_{80°C}$-values of each sample at month 0 and month 12 ($p < 0.05$). Uppercase letter shows the significant differences between $D_{80°C}$-values of each sample at $a_w = 0.25$ and $a_w = 0.50$. Mean ± SD, n = 3.

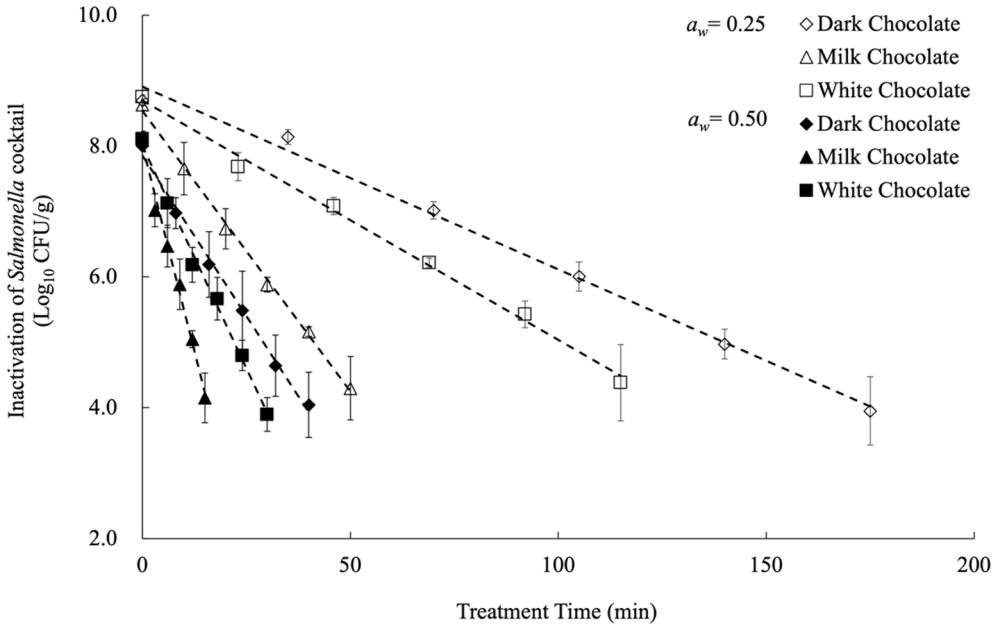

Fig. 4. The thermal inactivation kinetic curves of *Salmonella* in chocolate products with different a_w levels at 80 °C. Testing was conducted before storage, and a_w was measured at 22 °C, n = 3.

CRediT authorship contribution statement

Sicheng Sun: Conceptualization, Methodology, Investigation, Writing – original draft. **Yucen Xie:** Methodology, Writing – review & editing. **Xu Zhou:** 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.

Data availability

Data will be made available on request.

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