



Survivability of *Salmonella* and *Enterococcus faecium* in chili, cinnamon and black pepper powders during storage and isothermal treatments

Yucen Xie^a, Shuang Zhang^{a,b}, Sicheng Sun^a, Mei-Jun Zhu^c, Shyam Sablani^a, Juming Tang^{a,*}

^a Department of Biological Systems Engineering, Washington State University, Pullman, WA, 99164, USA

^b College of Mechanical and Electronic Engineering, Northwest A&F University, Yangling, 712100, China

^c School of Food Science, Washington State University, Pullman, WA, 99164, USA

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ABSTRACT

Outbreaks and recalls associated with foods containing spices suggest a need for risk assessment of *Salmonella* in spices. In this study, the survivability of *Salmonella* Enteritidis PT 30, *Salmonella* cocktail (S. Enteritidis PT 30, S. Tennessee K4643 and S. Agona 447967), and *Enterococcus faecium* NRRL B-2354 in chili, cinnamon and black pepper at water activities (a_w) 0.3 and 0.5 were evaluated during one-year storage at 21 °C. The thermal resistance of *Salmonella* cocktail in spices was also evaluated at 70 °C before and after storage. At a_w 0.5, 4-month storage caused 5 log reduction of *Salmonella* cocktail in chili, while 8 months led to the same level of reduction in cinnamon. But only 3 log reduction were observed in black pepper over one year. Storage at a_w 0.3 caused less reduction in *Salmonella* cocktail during the same storage periods. Less than 2 log reduction of *E. faecium* were observed over the one year storage at both a_w levels, except for in chili stored at 0.5 a_w . The $D_{70^\circ\text{C}}$ -values for *Salmonella* cocktail in chili, cinnamon and black pepper of a_w 0.3 before storage were 15.4, 20.8 and 36.6 min, respectively. 21–50% drops in the $D_{70^\circ\text{C}}$ -value were obtained after two-month of storage, mostly in chili and least in black pepper. The high $D_{70^\circ\text{C}}$ -value in black pepper persisted over one-year storage. Based on these results, chili powder showed the highest antimicrobial effect, followed by cinnamon and black pepper powders during storage and isothermal treatments.

1. Introduction

Spices are ready-to-eat low-moisture food ingredients used worldwide in the food industry and daily cuisine. Chili, cinnamon and black pepper are the most popular spices used in the United States and other countries (Van Doren et al., 2013). Spices are generally defined as aromatic vegetable substances used as seasoning rather than nutrition (FDA, 1980). Due to cultivation limitations, most of the spices consumed in the United States are imported from countries with tropical climates, including Indonesia, India, China and Mexico (Buzzanell et al., 1995).

Salmonella is one of the most prevalent microbial pathogens in spices associated with product recalls and foodborne illness outbreaks (CFRAN and FDA, 2017; The American Spice Trade Association, 2017). For example, in 1993, a salmonellosis outbreak associated with 1000 cases occurred in Germany due to the contamination of paprika-powdered potato chips (Lehmacher et al., 1995). Between 2009 and 2010, 272 individuals from 44 states in the United States were infected with *Salmonella* Montevideo due to the consumption of salami associated with

contaminated black and red pepper (CFRAN and FDA, 2017). From 2000 to 2012, the prevalence of *Salmonella* in chili, cinnamon and black pepper at different points in the farm-to-table continuum surveillance was reported globally, including in Australia, Brazil, Ireland, Japan, Turkey, United Kingdom and the United States (CFRAN and FDA, 2017).

Pathogen reduction treatments such as steaming, irradiation and fumigation are often implemented as a part of the post-harvest processing of spices (Duncan et al., 2017; The American Spice Trade Association, 2017). However, these treatments may not be the final steps before delivery to consumers. Contamination may occur during drying (to remove the absorbed water during steam treatments), grinding (e.g., to produce cinnamon powder from steam-treated cinnamon sticks), and packaging. Thus, pathogen reduction measurement needs to be included in the whole supply chain. Spices are typically stored at ambient temperature for a period of time. Therefore, knowledge of the stability of pathogens during storage is essential to profile the risk assessment in spices.

Spice products have a water activity (a_w) normally ranging from 0.3

* Corresponding author.

E-mail address: jtang@wsu.edu (J. Tang).

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to 0.6 at room temperature. Such low a_w environments are below the threshold for microbial growth. However, foodborne pathogens such as *Salmonella* may survive under desiccated conditions for an extended period. A previous study on the survival of a three-strain *Salmonella* cocktail in almond meal (without antimicrobial property) reported only 0.8 and 1.5 log₁₀ CFU/g reduction at a_w 0.25 and 0.45, respectively, over one-year storage at 22 °C (Zhu et al., 2021). On the other hand, many spices have been documented to have natural antimicrobial attributes and can be used as additive ingredients to preserve other foods (Fenwick et al., 1985). The antimicrobial compounds present in spices are able to inhibit the growth of *Salmonella* and also influence its survivability in foods when the concentration of antimicrobial compounds is sufficient or the contamination level is not high (Arora & Kaur, 1999; Ceylan & Fung, 2004; Dorman & Deans, 2000; Weerakkody et al., 2011). The variations in the concentrations and types of antimicrobial compounds in different spices may also influence survival of *Salmonella* in storage (Nazzaro et al., 2013). For example, a four-serovar *Salmonella* cocktail (S. Enteritidis, S. Oranienburg, S. Tennessee and S. Anatum) could persist over a year in ground black pepper but only survive for 88 days in dehydrated garlic under typical storage conditions at ~25 °C and ambient relative humidity (Keller et al., 2013; Zhang et al., 2017). However, there has been a general lack of data regarding the survivability of *Salmonella* in different spices as influenced by a_w during storage.

Enterococcus faecium NRRL B-2354, a non-pathogenic commensal bacterium in human intestines, has been used as a surrogate for *Salmonella* in validating thermal pasteurization processes for low-moisture foods (Alshammari et al., 2021; Chen et al., 2019; Liu et al., 2018; Newkirk et al., 2018; Ozturk et al., 2019; Pérez-Reyes, 2019; Wei, Vasquez, et al., 2021; Xu et al., 2020). The suitability of *E. faecium* as a surrogate for *Salmonella* for thermal process verification depends on the factors such as types of foods, a_w level, and process conditions (Dhowlaghar & Zhu, 2021; Xu et al., 2021). However, the study for the suitability of *E. faecium* as a surrogate of *Salmonella* in spice products during long-term storage is limited. In addition, Nazzaro et al. (2013) suggest that the antimicrobial activities of spices and their extracts are more effective against Gram-positive bacteria than Gram-negative bacteria. *E. faecium* (Gram-positive) and *Salmonella* (Gram-negative) have different cell structures and responses to desiccation stress, resulting in different survivability to the natural antimicrobial compounds from spices in dry environments (Nazzaro et al., 2013). Therefore, it is important to investigate the antimicrobial activity of spices against both *Salmonella* and *E. faecium* in storage.

Based on recent outbreaks and recalls associated with *Salmonella* in spices, we selected chili powder, ground cinnamon, and black pepper powder as the representative spices in this study. The objectives of this study were to: 1) determine the survivability of S. Enteritidis, three-serovar *Salmonella* cocktail, and *E. faecium* in the three spices at a_w 0.3 and 0.5 during one-year room-temperature storage; 2) evaluate the difference in survival between the single strain S. Enteritidis and three-serovar *Salmonella* cocktail, as well as the suitability of *E. faecium* as a conservative surrogate for *Salmonella* in the three spices during storage; 3) investigate the thermal resistance of *Salmonella* cocktail in a_w 0.30 samples at 70 °C before and after storage.

2. Materials and methods

2.1. Physicochemical properties of the spices

Chili powder, ground cinnamon and black pepper powder of a common brand were purchased from a retail source. The initial a_w values of these spice products at room temperature (~21 °C) were measured in triplicate using a water activity meter (Aqualab, Meter Group, Inc., Pullman, WA, USA). The proximate analyses and particle size distribution of the three spices were conducted by the Particle Technology Labs (Chicago, IL, USA) and Northern California Laboratory (Silliker, Inc.,

Salida, CA, USA), respectively. The proximate analyses included the determination of ash, carbohydrates, fat, moisture and protein contents.

2.2. Bacterial strains

For storage studies, a single strain of S. Enteritidis and a three-serovar cocktail of *Salmonella* were tested in parallel to investigate the difference in survivability between them. The single strain, S. Enteritidis PT 30 (originally isolated from raw almond), was obtained from Dr. Linda Harris, University of California, Davis. A three-serovar *Salmonella* cocktail was prepared by combining S. Enteritidis, S. Tennessee K4643 (originally isolated from peanut butter) and S. Agona 447967 (originally isolated from toasted oat cereal). S. Tennessee K4643 and S. Agona 447967 were kindly gifted by Dr. Nathan Anderson from FDA, Chicago, Illinois. These three strains were originally isolated from contaminated spices and other low-moisture foods. Additional storage studies were conducted using a single strain of *E. faecium*. The *E. faecium* NRRL B-2354 strain was obtained from the USDA Agricultural Research Service (USDA-ARS) in Peoria, IL. Strains were kept 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.

2.3. Culture preparation

The single-strain inoculum was prepared by growing each strain separately. A loop of the frozen culture was propagated in TSBYE at 37 °C for 24 h and then sub-cultured in TSBYE for an additional 24 h at 37 °C. One mL of the twice-activated culture was then plated on a 150 × 15 mm tryptic soy agar (TSA, Difco™, Detroit, MI, USA) supplemented with 0.6% (w/v) yeast extract (TSAYE) and incubated at 37 °C for 24 h (in triplicate for each strain). The bacterial lawn was harvested from the three plates using ~40 mL sterile buffered peptone water (BPW), collected in a 50 mL conical tube (Falcon, Fisher Scientific), and then centrifuged at 6000×g, 4 °C for 15 min. The supernatant was discarded, and the residual pellet was resuspended in 1 mL sterile BPW to achieve ~10¹¹ CFU/mL for each inoculum of *Salmonella* or *E. faecium*. To create a 3-strain cocktail of *Salmonella*, an equal volume (1 mL) of each *Salmonella* serotype was combined into a conical tube and mixed together. The populations of S. Enteritidis, *Salmonella* cocktail or *E. faecium* inoculum were confirmed by plating appropriate dilutions on TSAYE and incubating at 37 °C for 24 h before enumeration.

2.4. Inoculation and preparation for storage studies

Each of the above three inocula were mixed into the spice sample at a ratio of 1 mL (inoculum): 100 g (spice) for the storage tests. In brief, 5 g of each spice sample was first inoculated with 1 mL of bacterial inoculum (~11 log₁₀ CFU/mL) and mixed manually in a 4 oz. Whirl-Pak® bag (Nasco™, FisherScientific) for up to 5 min leading to no visible clumps. Then, 5 g more of uninoculated spice was added into the same bag. The samples were manually massaged and then stomached at 230 rpm for 3 min (Stomacher® 400 Circulator, Seward Laboratory System Inc., Norfolk, UK). Each of the above homogenous inoculated samples (~10 g) was further transferred into a 10 oz. Whirl-Pak® bag, along with ~90 g of uninoculated spice. The mixture was manually shaken and massaged multiple times to achieve homogeneity. Detection of the background flora for spice products was conducted by plating several appropriate dilutions of spices on TSAYE and incubating at 37 °C for 24 h.

Each inoculated sample (~100 g) was randomly divided and spread onto four 150 × 15 mm Petri dishes and air-dried in a bio-safety hood (ambient relative humidity, RH, 30–34% and temperature ~21 °C) to reach appropriate a_w levels (0.3 and 0.5). The two a_w levels were selected based on the common range of spice products. In this study, the initial a_w values of the inoculated spice samples were typically about 0.6;

a half-hour of air drying could roughly reduce the a_w to 0.5. After an additional one and half-hour (2 h in total), the a_w of the sample could reach about 0.3. The Petri dishes were then placed in a humidity/temperature-controllable chamber (Mettler HCP 50 humidity chamber, Germany) for an additional two days to ensure that the a_w reached 0.30 or 0.50 ± 0.02 , respectively. The equilibrated samples were collected from four Petri dishes into a 10 oz. Whirl-Pak® bag and manually mixed for 5 min. The a_w of the above-conditioned samples (slightly less than 100 g) was confirmed in triplicate using a water activity meter (AquaLab, Meter Group, Inc., Pullman, WA). The initial inoculation level and its population homogeneity were also confirmed by enumeration from 5 randomly selected sub-samples.

2.5. Microbial survivability in spices during room-temperature storage

The above-conditioned samples (three types of spices, two a_w levels, and three inoculums) were split into 2 oz. Whirl-Pak® bags (~3 g each), and hermetically sealed in several moisture barrier bags constructed from layers of nylon, foil and polyethylene (Dri-Shield 3000®, Desco Industries, Inc) to create a storage environment similar to that for commercial spice products. These packed samples were stored at room temperature (~21 °C). The packs were sampled for survival study every week in the initial two months and then tested monthly or bimonthly until the end of one-year of storage. The a_w of samples was also monitored at each sampling point. Two biologically independent replicates were prepared for each condition, and three technical replicates were carried out for each independent replicate.

2.6. Thermal resistance of salmonella cocktail in spices and its change during storage

2.6.1. Isothermal inactivation studies

The isothermal inactivation studies were conducted for the *Salmonella* cocktail in the three spices at 70 °C before and after storage. In order to yield meaningful results from the isothermal inactivation tests, an initial population of more than 10^6 CFU/g in the spices is required. Our preliminary tests showed sharp reduction of *Salmonella* cocktail in a_w 0.5 chili powder in short time storage. Thus, the comparative isothermal inactivation tests were only conducted on a_w 0.3 spices before storage and after two-month of storage for the three spices, and after one-year of storage for black pepper. According to Xie et al. (2021), spices with antimicrobial activities reduced the thermal resistance of *S. Enteritidis* during isothermal treatments. They reported similar *D*-values of *S. Enteritidis* in ground cinnamon when heated at 70 °C as that in wheat flour and other low-moisture foods when treated at 80 °C (Xie et al., 2021). Thus, the investigation for the thermal resistance of *Salmonella* cocktail in chili, cinnamon and black pepper in this study was carried out at 70 °C.

The closed system test method is one of the most appropriate ways to study thermal inactivation of pathogen control in low moisture foods (Cheng et al., 2021). The improved Thermal-Death-Time test cell (TDT II) was used in this study. The TDT II test cells has a smaller come-up-time (CUT, min) because of a larger thermal contact surface and a smaller sample depth as compared to the previous design of TDT cells (Chung et al., 2008; Jin & Tang, 2019). CUT is the time required for the sample to reach the target temperature within 0.5 °C (69.5 °C in this study), and it was measured in a TDT II cell with a 0.5 mm-diameter thermocouple placed through the center of its lid. The measurement of CUT in spices was conducted according to a previously published method (Xie et al., 2021). Briefly, about 0.6 g of spice sample was added to the above specially designed test cell, hermetically sealed, and then immersed in a pre-heated glycol oil bath with circulator (Isotemp™ 5150 H24, Fisherbrand™, PA, USA). The sample temperature was recorded using a data logger. In this study, the CUT for the three spices to reach the target thermal-treatment temperature (69.5 °C) varied from 54 s to 1 min 12 s. To simplify data analyses, the CUT for the isothermal

treatments of the three spices was considered to be 1 min.

Samples were inoculated with the *Salmonella* cocktail and conditioned using the same protocol as described in section 2.4. Briefly, 0.60 ± 0.01 g of conditioned spice sample (a_w 0.3) was added into each TDT II cell and sealed. The test cells were then subjected to a pre-heated oil bath at 70 °C for isothermal kinetic tests. The isothermal treatment time started recording after the CUT (regarded as 0 min in thermal death curves). The test cells from the respective treatment were collected at five sampling points (in duplicates at each point) and immediately cooled by immersing in ice water for 2 min. All isothermal treatments for spice samples at month 0, month 2 and month 12 were repeated in two biological replicates.

2.6.2. Enumeration of viable bacteria in thermally treated spices

Thermally treated samples from each test cell (~0.6 g) were mixed in a 15-mL sterile conical centrifuge tube (Corning™ Falcon™, FisherScientific) with 5.4 mL of sterile BPW with 0.5% (w/v) potassium sulfite (K_2SO_3 , Acros Organics) to achieve a 10-fold dilution, then homogenized using a vortex for 1 min and further serially diluted to appropriate levels. The purpose of K_2SO_3 is to neutralize the antimicrobial effect of spices during enumeration (Lins, 2018b). The appropriate dilutions were spread on TSAYE plates in two technical replicates and incubated at 37 °C for 24 h.

2.6.3. Thermal inactivation parameters

The first-order kinetic model was applied for the analyses of the isothermal inactivation data, which can be expressed as Eq. (1) (Peleg, 2006):

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

where t (min) is the thermal treatment time; N (CFU/g) is the bacterial population at treatment time t ; N_0 (CFU/g) is the initial bacterial population at the CUT in this study; and D (min) is the time required to inactivate the microbial population by 90% at the treatment temperature. Thus, the thermal decimal time (*D*-value) was estimated from the negative reciprocal for the slope of the thermal inactivation curve using a log-linear regression; the goodness of fit was evaluated by R^2 coefficient.

2.7. Statistical analyses

The survivability curves and thermal death curves were plotted by the Prism 8 program (GraphPad Software, San Diego, CA). P-values of less than 0.05 were considered significant.

3. Results and discussion

3.1. Chemical composition and particle size distribution of the spices

The initial a_w of chili, cinnamon and black pepper powders at ~21 °C was 0.50 ± 0.02 , 0.51 ± 0.02 , and 0.53 ± 0.02 , respectively. The chemical composition for cinnamon, chili and black pepper powders are shown in Table 1. Spices can be considered carbohydrate-rich low-

Table 1
Chemical composition of chili, cinnamon and black pepper powders.

	Chili	Cinnamon	Black Pepper
Moisture (g water/100 g sample)	8.2 ± 2.9	9.6 ± 0.1	9.9 ± 0.2
Protein (% w/w)	17.9 ± 0.1	3.8 ± 0.2	12.9 ± 0.2
Fat (% w/w)	12.9 ± 0.6	2.4 ± 0.1	4.8 ± 0.1
Ash (% w/w)	9.0 ± 0.1	3.4 ± 0.1	3.9 ± 0.0
Carbohydrate (% w/w)	52.0 ± 3.6	80.7 ± 0.3	68.0 ± 0.8

Data for cinnamon was derived from Xie et al. (2021). Mean \pm SD; for chili and black pepper, $n = 2$.

moisture foods. Ground cinnamon had the highest carbohydrate content (80.7%), followed by black pepper powder (68.0%), and then chili powder (52.0%), as shown in Table 1. Chili and black pepper powders had relatively larger protein contents, accounting for 17.9% and 12.9%, respectively, compared to only 3.8% in cinnamon. In addition, the fat content in chili powder was 12.9%, while it was 2.4% and 4.8% in cinnamon and black pepper. This may be due to the high presence of chili seeds which are considered a source of crude fat/oil (Wang et al., 2014).

The particle size distribution or the granularity of the spices determines specific surface area exposure to the bacteria which may influence the rate of release of antimicrobial constituents (Kuang et al., 2011). Thus, the particle size distribution of chili, cinnamon and black pepper powders were measured. All three spices had broad ranges of particle size as shown in Fig. 1. In chili powder, a large proportion of the particles was between 180 and 250 μm (40.8%). The particles of cinnamon powder were evenly distributed between 75 and 355 μm (from 16.6 to 19.7%). For black pepper powder, the majority of particles were distributed around 425 μm (35.0%). Overall, the average particle size of the three spice products followed the order: black pepper powder > chili powder > ground cinnamon. About 65%, 73% and 100% of the particles in black pepper, chili and cinnamon were distributed between 75 and 425 μm , respectively.

3.2. Microbial survivability in spices during room-temperature storage

The initial populations for the inocula of *S. Enteritidis*, *Salmonella* cocktail and *E. faecium* were 11.6 ± 0.1 , 11.3 ± 0.0 and $10.9 \pm 0.2 \log_{10}$ CFU/mL, respectively. Regardless of the a_w and strain, the initial populations of the inoculated spices at the starting point of storage (after conditioning, month/week 0) were between 7.5 and 9.1 \log_{10} CFU/g with standard deviations less than 0.5 \log_{10} CFU/g. The fluctuation of a_w of the packed samples varied from 0.01 to 0.04 for all spices during the one-year storage.

3.2.1. Survival of *S. Enteritidis* and three-serovar *Salmonella* cocktail in spices

S. Enteritidis populations in the three spices at a_w 0.3 and 0.5 over the first 8-weeks of storage are illustrated in Fig. 2A. The population of *S. Enteritidis* in chili powder gradually dropped with storage time. Less reduction was observed at a_w 0.3 than at a_w 0.5. At the end of the eighth week, the population was reduced by 1.4 and 4.0 \log_{10} CFU/g in a_w 0.3 and 0.5 chili powder, respectively. In cinnamon powder, *S. Enteritidis*

was relatively stable over the initial 4-weeks of storage at both a_w levels, but the population dropped by 1.0 and 1.3 \log_{10} CFU/g at a_w 0.3 and 0.5 after the 8-week storage, respectively. For black pepper, microbial populations at a_w 0.3 were $\sim 0.5 \log_{10}$ CFU/g higher than that at a_w 0.5, but they were stable over the entire 8 weeks of storage (Fig. 2A).

The decline of *S. Enteritidis* populations in the three spices was more evident from the test results for one-year storage (Fig. 3A). The rate of reduction depended on the type of spice and the a_w . Consistent with results from the initial 8-weeks of storage, the highest reduction was observed in chili powder. Specifically, 8-month storage caused more than 6 log reduction of *S. Enteritidis* in a_w 0.3 chili, while 3-month storage led to a similar level of population drop in a_w 0.5 chili. *S. Enteritidis* counts declined by 5.5 and 5.6 \log_{10} CFU/g, respectively, in a_w 0.3 and 0.5 cinnamon powder over a year, in which the influence of a_w was not significant. In black pepper, the *S. Enteritidis* population dropped by 1.2 \log_{10} CFU/g at a_w 0.3 after 12 months and dropped by 3.4 \log_{10} CFU/g at a_w 0.5 after the same period of storage. Keller et al. (2013) also reported 3 log reduction of four-serovar *Salmonella* cocktail (including *S. Enteritidis*, *S. Oranienburg*, *S. Tennessee* and *S. Anatum*) in black pepper after one-year storage at 40% RH (equivalent to product a_w of 0.4) and 25 °C. This is in between our results for black pepper powder stored at a_w 0.3 and 0.5.

Survival of the three-serovar *Salmonella* cocktail during the initial 8-week of storage at room temperature is illustrated in Fig. 2B. Different declines of the *Salmonella* cocktail populations were observed in chili and cinnamon powders. After 8 weeks, 1.0 and 2.2 \log_{10} CFU/g of *Salmonella* cocktail drops were seen in a_w 0.3 and 0.5 chili powder, respectively. In ground cinnamon, the counts of *Salmonella* cocktail reduced by 0.9 and 1.2 \log_{10} CFU/g in a_w 0.3 and 0.5, respectively, after 8-weeks of storage. However, the population remained stable in black pepper powder over the 8-week storage period (Fig. 2B). Similar to *S. Enteritidis*, the *Salmonella* cocktail showed a higher stability in the three spices under drier conditions, resulting in less population decline observed in samples at a_w 0.3 compared to that at a_w 0.5.

The data for *Salmonella* cocktail survival in the three spices during one year of storage are shown in Fig. 3B. Specifically, after storage at a_w 0.5 for 4 months, about 5.1 \log_{10} CFU/g reduction was obtained in chili powder, followed by ground cinnamon with 2.4 log reduction. But only 0.7 \log_{10} CFU/g of *Salmonella* cocktail was observed in a_w 0.5 black pepper powder after 4 months' storage. Once again, smaller population drops were obtained in the three spices at a_w 0.3. The counts of *Salmonella* cocktail declined by 4.7, 4.4 and 0.8 \log_{10} CFU/g in chili, cinnamon and black pepper at a_w 0.3 after the one-year storage. Compared with

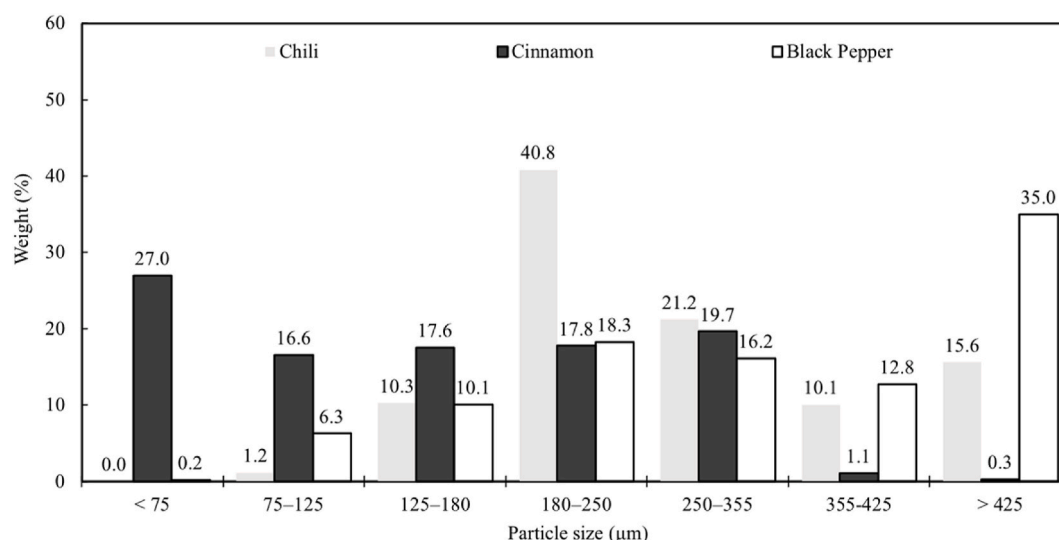


Fig. 1. Particle size distribution of chili, cinnamon and black pepper. Mean \pm SD, $n = 2$.

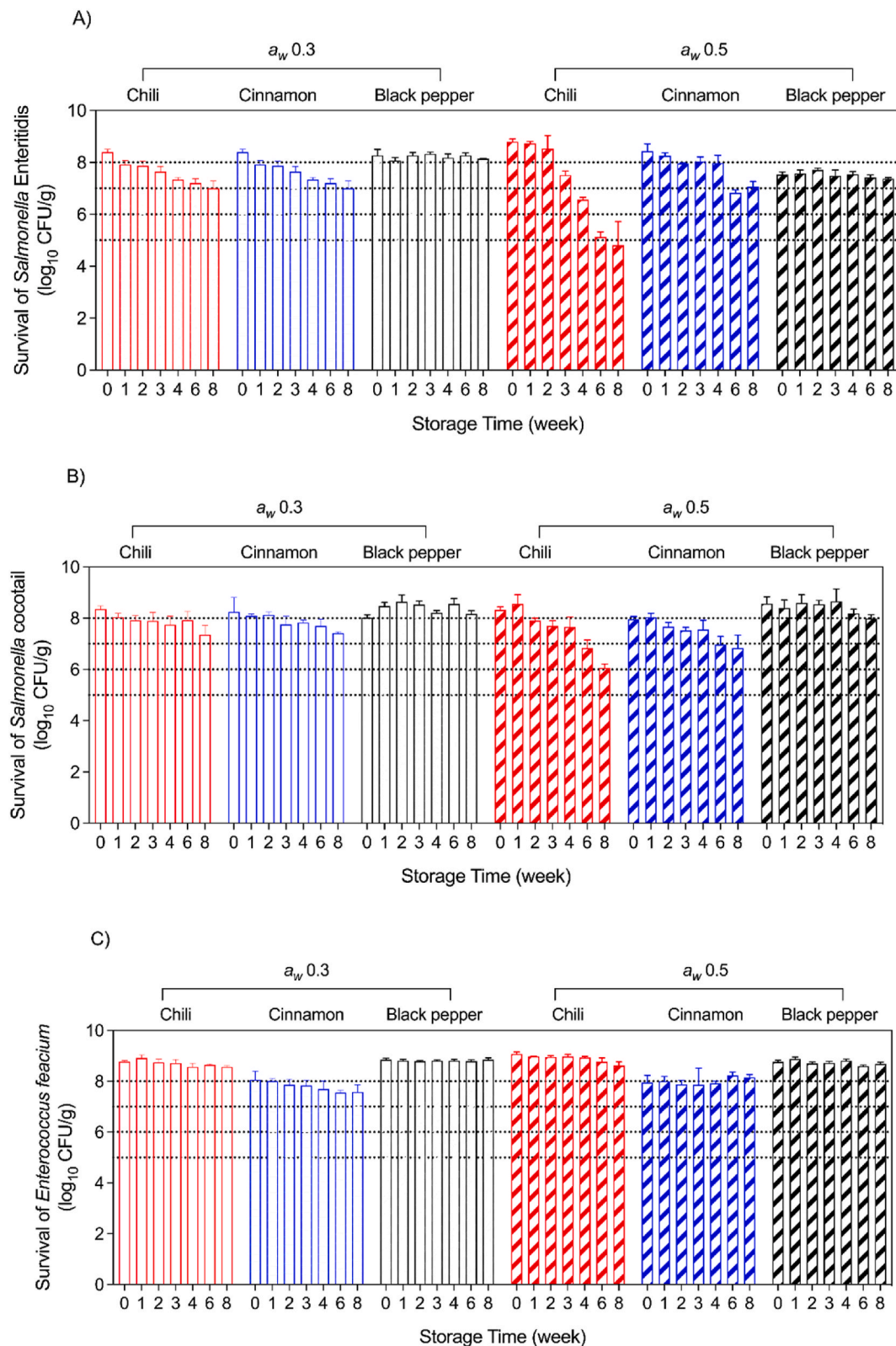


Fig. 2. The survival of (A) *S. Enteritidis*, (B) *Salmonella* cocktail and (C) *E. faecium* in chili (red bars), cinnamon (blue bars) and black pepper (black bars) at two a_w levels (0.3 and 0.5) over the initial 8-week storage at room temperature. Mean \pm SD; $n = 3$ for each biological replicate and experiments were carried out in duplicate.

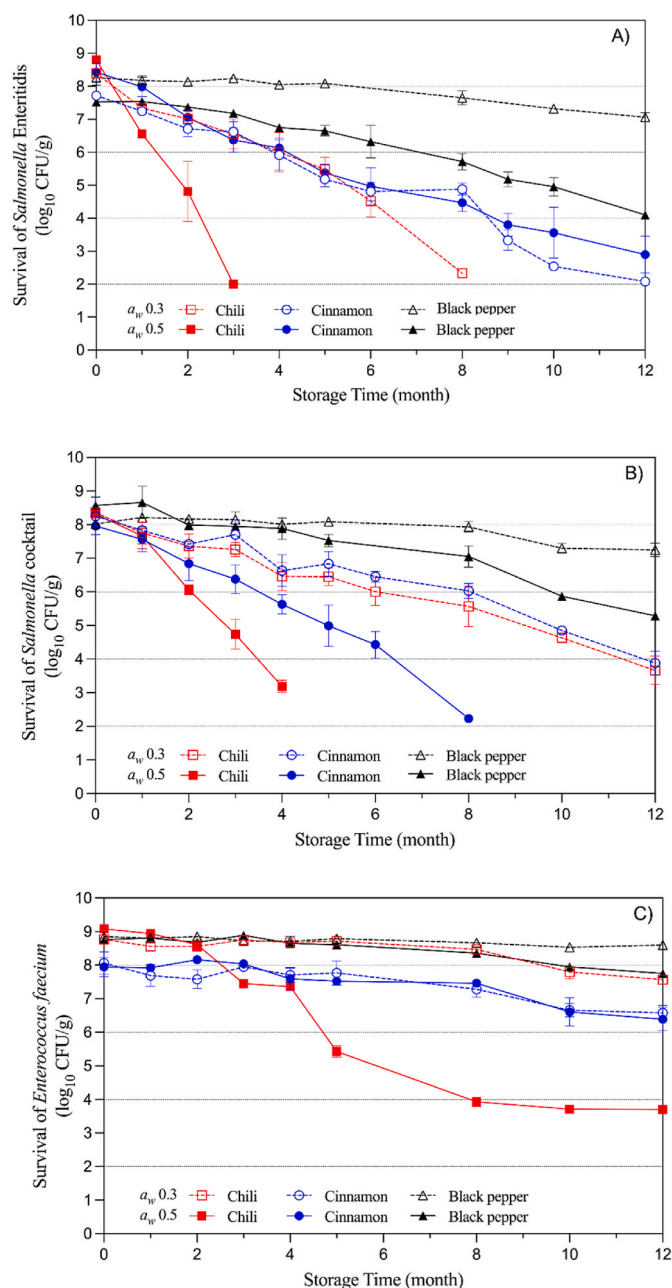


Fig. 3. The survival of (A) *S. Enteritidis*, (B) *Salmonella* cocktail and (C) *E. faecium* in chili (red symbols), cinnamon (blue symbols) and black pepper (black symbols) at two a_w levels (0.3 and 0.5) over a 12-month storage period at room temperature. Open symbols represent the sample at a_w 0.3 and closed symbols refer to the sample at a_w 0.5. Mean \pm SD; $n = 3$ for each biological replicate and experiments were carried out in duplicate.

low-moisture foods without antimicrobial properties, such as almond flour, *Salmonella* are less stable in certain spices during storage. For instance, a reduction of 0.8 and 1.5 log₁₀ CFU/g of the same three-strain *Salmonella* cocktail were observed in a_w 0.25 and 0.45 almond flour after one-year storage at room-temperature (Zhu et al., 2021). The population reductions in chili and cinnamon powder are significantly larger than that in almond flour, but the decline in black pepper is close to that in almond flour.

The survival of *Salmonella* cocktail was higher than the single strain *S. Enteritidis* in chili and black pepper at both a_w levels during storage. Similarly, the reduction of *Salmonella* cocktail was smaller than the single strain *S. Enteritidis* in cinnamon at a_w 0.3. However, *S. Enteritidis*

had higher survivability than the *Salmonella* cocktail in cinnamon at a_w 0.5. The three-strain cocktail included the *S. Enteritidis*, *S. Tennessee* and *S. Agona*. The different survival of single strain and three-strain cocktail in the three spices (Fig. 3A and B) indicate that the serotypes of *Salmonella* influenced their survival under same dry conditions, similar to the findings reported in Farakos et al. (2014). It was suggested that *Salmonella* cells could acquire cross-protection to multiple stressors in low- a_w environments (Finn et al., 2013; Gruzdev et al., 2011).

In this study, chili was the most effective spice against *Salmonella* during room-temperature storage, particularly at a_w 0.5. A similar observation was previously reported in a storage study on *S. Oranienburg* survivability in 9 spices and herbs, including chili, cinnamon and black pepper (Lins, 2018a). According to Lins (2018b), *S. Oranienburg* showed 6 log₁₀ CFU/g reduction in chili after storage for 6 months at 25 °C; while only 2 and 1 log reduction were observed in cinnamon and black pepper, respectively, after storage for one year. But the a_w of the samples was not reported in Lins' study. As shown in the results of our studies discussed above, the value of a_w is an important factor influencing the microbial survivability. The controlled a_w in the three spices in our studies provided equivalent desiccation stress to *Salmonella* during storage. Our results verified and reinforced the general conclusion of Lins' study that chili powder had a more significant antimicrobial effect against *Salmonella* during storage when compared to cinnamon or black pepper. It also suggests that *Salmonella* had greater stability in black pepper when compared to cinnamon during the storage with equivalent a_w . With controlled a_w of samples, our results provide reliable information regarding microbial stability in cinnamon and black pepper.

Based on the *Salmonella* survivability during storage, the antimicrobial effectiveness of the three spices is in the following order: chili > cinnamon > black pepper. The exact antimicrobial mechanisms of spices and their components against pathogens are not well understood. Temperature, pH, the amount of oxygen, antimicrobial composition, concentration and the synergistic effect of multiple antimicrobial agents are reported to determine susceptibility towards pathogens (Burt, 2004; Gutierrez et al., 2008; Morrone et al., 2018; Nazzaro et al., 2013; Vasconcelos et al., 2018). Further studies are needed to characterize and quantify antimicrobial composition in spices to better understand their antimicrobial activities against pathogens.

3.2.2. Survival of *E. faecium* in spices

The survival of *E. faecium* over the initial 8 weeks and one year are illustrated in Figs. 2C and 3C, respectively. More *E. faecium* reduction was observed in ground cinnamon during the conditioning prior to the storage, resulting in a lower *E. faecium* population in cinnamon than the other two spices at the starting point of storage. Regardless of the a_w , the population of *E. faecium* in the three spices remained stable over the initial 8 weeks; no significant population drop was observed (Fig. 2C). The counts of *E. faecium* were relatively stable in the three spices at a_w 0.3. After 12 months, *E. faecium* populations dropped by 1.2, 1.5 and 0.2 log₁₀ CFU/g, respectively, in a_w 0.3 chili, cinnamon and black pepper. At a_w 0.5, the population of *E. faecium* in chili powder started to drop after 2 months and declined by 5.4 log₁₀ CFU/g after one year (Fig. 3C). But only about 1 and 1.5 log reduction were observed in a_w 0.5 cinnamon and black pepper after 12-month storage at room temperature.

The above storage results indicate that *E. faecium* was more stable than *S. Enteritidis* and *Salmonella* cocktail in the three spices during room-temperature storage (Fig. 3A, B and C). *E. faecium* also shows a higher desiccation tolerance than *Salmonella* during storage in other low moisture foods, such as peanut paste formulations (Kataoka et al., 2014) and milk powder (Wei, Agarwal, & Subbiah, 2021). Studies have shown that the antimicrobial reaction of spices and spices extracts are more effective on the inhibition of Gram-positive bacteria than Gram-negative bacteria (Nazzaro et al., 2013). However, in this study, the survival of *E. faecium* as a Gram-positive bacteria was more stable than the Gram-negative *Salmonella* in the three spices during storage. The antimicrobial mechanisms may be different in the growth and survival of

bacterial cells.

3.3. Suitability of using storage as a pathogen control for low moisture spices

Storage is a crucial link in the spice production chain. According to the American Spice Trade Association (ASTA), two appropriate storage periods are included in the supply chain: one is prior to pathogen reduction treatment and another is before retail sales (The American Spice Trade Association, 2017). Our storage tests may provide information for the industry to estimate appropriate storage periods of some spice products to ensure microbial safety. In this study, *Salmonella* could survive at a_w 0.5 in the three spices for at least 3 months and up to one year; while at a_w 0.3, *Salmonella* persisted for at least 8 months. Except for the a_w 0.5 chili powder, the populations of *E. faecium* remained at high levels in samples after 12 months at both a_w .

In the validation of pathogen control for food products, including spices, it is generally required to achieve a minimal 5-log reduction of *Salmonella* (The American Spice Trade Association, 2017). The a_w values of receiving cinnamon, chili and black pepper products are all close to 0.5 (as mentioned in section 3.1). Taking the *Salmonella* cocktail in a_w 0.5 spices as an example, it needs 4 and 8 months to obtain a 5-log reduction in chili and cinnamon, respectively; however, only 3 log reduction were observed in black pepper after one year. Therefore, 4 months of ambient-temperature storage for chili products with potential *Salmonella* contamination may be enough to eliminate the safety risks without pasteurization, thus saving cost for the manufacturers. But for some other spices, such as black pepper, a decontamination process may be needed to ensure safety.

Increasing storage temperature may be an alternative way to improve the inactivation during storage. Several studies related to spice products demonstrated that higher storage temperatures would facilitate the inactivation of pathogens (Adler & Beuchat, 2002; Keller et al., 2013; Zhang et al., 2017). For example, *Salmonella* was able to survive up to 73 days in dehydrated garlic flakes at 25 °C under ambient RH, while the population immediately dropped below the detection limit after 9 days at 35 °C under the same RH (Zhang et al., 2017). Thus, for spices such as black pepper with small population drops during room-temperature storage, more declines might be achieved at an increased temperature during storage. Keller et al. (2013) concluded that *Salmonella* population in black pepper had a larger reduction when stored at 35 °C than at 25 °C under the same RH. Similar observations were also reported for *Salmonella*, *Listeria monocytogenes*, *Escherichia coli* and *Cronobacter sakazakii* in low moisture foods without antimicrobial activity, including peanuts (Brar et al., 2015), walnuts (Blessington et al., 2012), powdered infant formula (Koseki et al., 2015), almond and pistachios (Kimber et al., 2012). The increased temperature may contribute to more release of antimicrobial compounds in spices.

3.4. Thermal inactivation of *Salmonella* cocktail in the three spices

In analyzing the thermal inactivation data for *Salmonella* cocktail in the three spices using Eq. (1), the CUT for the isothermal treatments at 70 °C was normalized to 1 min. At the CUT, populations of *Salmonella* cocktail in the three spices were of the same order of magnitude ($\sim 8 \log_{10}$ CFU/g). The thermal death curves for the *Salmonella* cocktail in the three spices (a_w 0.3) were generated by plotting the survival populations at different sampling times (Fig. 4).

Generally, the reduction of *Salmonella* cocktail in all three spices decreased linearly with treatment time on a semi-log scale ($R^2 \geq 0.98$). The inactivation rates of *Salmonella* cocktail depended on spices. When samples were thermally treated for 60 min at 70 °C, the counts of *Salmonella* cocktail declined by ~ 4 , 3, and less than 2 \log_{10} CFU/g in chili, cinnamon and black pepper, respectively (Fig. 4). The three samples had the same a_w 0.3 at room temperature, and more population drops were observed in chili than the other two spices for the same treatment time,

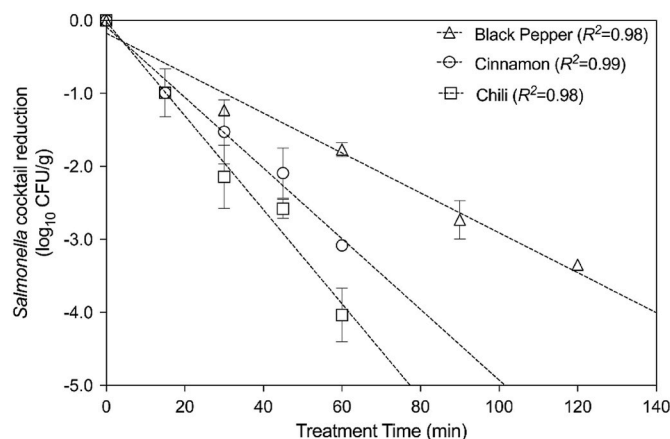


Fig. 4. Thermal death curves of *Salmonella* cocktail in the three spices at a_w 0.3, 70 °C, before storage (month 0). Mean \pm SD; $n = 2$ for each biological replicate and experiments were carried out in duplicate.

suggesting that chili powder was more effective against *Salmonella* cocktail during thermal inactivation. The antimicrobial effectiveness for the three spices on the *Salmonella* cocktail during thermal inactivation is consistent with the conclusion in the above storage tests.

The factors influencing the survival of *Salmonella* in low-moisture foods during thermal treatment include treatment-temperature a_w or moisture content (Xie et al., 2020; Xu et al., 2019), bacterial strain/-serotype (He et al., 2013; Li et al., 2014), and food composition (Jin et al., 2019; Li et al., 2014). A thermal inactivation study for the single strain *S. Enteritidis* PT30 in ground cinnamon was reported by Xie et al. (2021). The $D_{70^\circ\text{C}}$ -values for *S. Enteritidis* PT30 in a_w 0.3 cinnamon powder was 15.3 min, which is slightly smaller than that for *Salmonella* cocktail in cinnamon under the same thermal treatment conditions used in this study (20.8 min, Table 2). This comparison indicates that variability in strains or serotypes (single strain versus 3-strain cocktail) led to different thermal resistance. Another thermal inactivation study for the same 3-strain *Salmonella* cocktail was tested in cocoa powder (Tsai et al., 2019). When the a_w 0.3 cocoa powder was thermally treated at 70 °C for 60 min, only about 1.3 log reduction was observed. The $D_{70^\circ\text{C}}$ -value for *Salmonella* cocktail in a_w 0.3 cocoa powder (46.2 min) was close to that in black pepper before long-term storage (36.6 min, Table 2).

3.5. The change of $D_{70^\circ\text{C}}$ -values for *Salmonella* cocktail in spices after storage

The $D_{70^\circ\text{C}}$ -values for *Salmonella* cocktail in the three spices (at a_w 0.3) before and after storage are summarized in Table 2. The $D_{70^\circ\text{C}}$ -values in

Table 2

The changes of $D_{70^\circ\text{C}}$ -values for *Salmonella* cocktail in the a_w 0.3 spices during storage.

Spice	$D_{70^\circ\text{C}}$ values (min)					
	Month 0	R^2	Month 2	R^2	Month 12	R^2
Chili	15.4 \pm 1.6 ^a (100%)	0.99	7.7 \pm 0.4 ^b (50%)	0.96	–	–
Cinnamon	20.8 \pm 0.4 ^a (100%)	0.98	8.8 \pm 2.0 ^b (42%)	0.89	–	–
Black pepper	36.6 \pm 0.6 ^a (100%)	0.98	28.9 \pm 0.5 ^b (79%)	0.97	27.3 \pm 1.6 ^b (75%)	0.90

The values in the parentheses were the percentage for the $D_{70^\circ\text{C}}$ -values after storage (month 2 or 12) to the $D_{70^\circ\text{C}}$ -values before storage (month 0). Isothermal treatments were carried out in two biological replicates with two technical replicates. Different lowercase letters for each row indicate the significant difference ($P < 0.05$). Mean \pm SD.

chili, cinnamon and black pepper before storage (at month 0) were 15.4 ± 1.6 , 20.8 ± 0.4 and 36.6 ± 0.6 min, respectively. After two months of storage at room temperature, the $D_{70^\circ\text{C}}$ -values dropped to 7.7 ± 0.4 , 8.8 ± 2.0 and 28.9 ± 0.5 min, respectively. These drops in D -values for the three spices after 2 months are all statistically significant ($P < 0.05$). The $D_{70^\circ\text{C}}$ -values in a_w 0.3 chili and cinnamon measured at month 2 were about half that in the same samples at month 0 (dropped by 50% and 58%, respectively). In a_w 0.3 black pepper, the $D_{70^\circ\text{C}}$ -value showed an initial drop of 21% after 2 months, and then remained stable for over 12 months ($P > 0.05$).

According to the literature, bacteria in low-moisture foods without antimicrobial activities tended to maintain their thermal resistance during extended storage under different a_w and temperatures (Ballom et al., 2020; Cheng & Wang, 2018; Taylor et al., 2018; Zhu et al., 2021). Regardless of storage conditions (a_w 0.25 or 0.45; 4 or 22 °C), the thermal tolerance of *Salmonella* in almond meal after one-year storage remained at the same level as that before storage (Zhu et al., 2021). Similar observations were also found for *Listeria monocytogenes* in not-fat milk powder and wheat flour (Ballom et al., 2020; Taylor et al., 2018) or *Escherichia coli* in almond powder (Cheng & Wang, 2018) during extended storage. As mentioned above, even though the reduction of the same *Salmonella* cocktail in almond flour and black pepper during storage were similar, the thermal resistance was significantly affected in black pepper. This comparison suggests that the bacterial cells in black pepper survived from the antimicrobial reaction during storage, but they might have been heavily injured and thus lose the tolerance to heat. After specific storage periods, *Salmonella* was no longer able to maintain its original thermal tolerance in spices compared with other low-moisture foods, indicating that the three spices may somehow damage the bacterial cells during storage and lead to the loss of heat tolerance.

4. Conclusions

The stability of *S. Enteritidis* PT30, three-serovar *Salmonella* cocktail and *E. faecium* in chili, cinnamon and black pepper powders were dramatically influenced by the a_w during room-temperature storage. *Salmonella*, including the single strain and the cocktail, could persist in the three spices for at least 3 months and up to one year, depending on a_w and type of spices. The lower a_w environment helped the bacteria survive in the three spices during extended storage. Different antimicrobial effectiveness of spices to bacterial stability was observed during room-temperature storage and thermal inactivation. In this study, the stability of *Salmonella* in the three spices followed the coincident order in the storage and isothermal treatments: chili > cinnamon > black pepper. Our data may provide insightful information to develop appropriate storage for chili and cinnamon products as an alternative cost-effective way to control potential safety risks. For instance, instead of thermal pasteurization, 4 and 8 months of storage can cause 5 log *Salmonella* reduction in chili and cinnamon product ($a_w \sim 0.5$), respectively. Unfortunately, one-year storage only led to a 3-log reduction in black pepper at a_w 0.5. Thus, a pathogen reduction step is still required for black pepper to ensure microbial safety within appropriate periods. The *E. faecium* showed higher survivability than *Salmonella* in the three spices during storage, indicating its suitability as a surrogate for *Salmonella* in spices for storage test. Further studies are needed to evaluate its suitability in spices during thermal inactivation. Spices are used as food ingredients with other food systems. Thus, future studies should also focus on the mixture of spices and other low-moisture foods to evaluate the antimicrobial effect of spices as additive ingredients to other food systems.

CRedit authorship contribution statement

Yucen Xie: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft. **Shuang**

Zhang: Investigation, Writing – review & editing. **Sicheng Sun:** Assistance in sample preparation and enumeration, 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 have no conflict of interest in this manuscript.

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