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FOOD MICROBIOLOGY

Long-Term Survival and Thermal Death Kinetics of Enterohemorrhagic *Escherichia coli* Serogroups O26, O103, O111, and O157 in Wheat Flour

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ABSTRACT Wheat flour has been associated with outbreaks of enterohemorrhagic *Escherichia coli* (EHEC), but little is known on EHEC's survival during storage and thermal processing. The objective of this study was to determine long-term viability and thermal inactivation kinetics of EHEC serogroups O26, O103, O111, and O157. Wheat flour samples were inoculated with a cocktail of five strains of a single serogroup and stored at 23 and 35°C. Inoculated samples were heated at 55, 60, 65, and 70°C. Viability was determined by plate counting. Decimal reduction time (D) and first decimal reduction time (δ) values were calculated with log-linear and Weibull models, respectively. At 23°C, EHEC counts declined gradually for 84 days and samples tested positive from 84 to 280 days. The thermal resistance (D and δ) values ranged from 7.5 to 8.2 and 3.1 to 5.3 days, respectively, but there were no significant differences among serogroups ($P \leq 0.05$). At 35°C, no EHEC was quantifiable by day 7 and no positive samples were detected after 49 days. Heating at 55 and 65°C resulted in δ-value ranges of 15.6 to 39.7 min and 3.0 to 3.9 min, respectively, with no significant difference among serogroups either. Z values were 12.6, 6.7, 10.2, and 13.4°C for O26, O103, O111, and O157, respectively. Thermal death kinetics of EHEC in flour were better described using the Weibull model. Survival and inactivation rates of four serogroups were remarkably similar. These findings indicated that all EHEC serovars tested remained viable for at least 9 months at room temperature and survived for up to 60 min at 70°C in wheat flour.

IMPORTANCE Enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella* have recently caused several gastroenteritis outbreaks and recalls of wheat flour. Because EHEC can cause illness with very low doses and there is very scarce information regarding their ability to survive storage and heating in flour, the present study was undertaken to assess the long-term survival of EHEC serogroups O26, O103, O111, and O157 in flour. These findings are relevant, as we report that EHEC can survive for more than 9 months in wheat flour during storage. In addition, results obtained suggest that thermal inactivation at 65°C for 30 min or 2 months of storage at 35°C may be feasible strategies to mitigate the risk of most EHEC serovars in wheat flour.

KEYWORDS cereal, enterohemorrhagic *E. coli*, *Escherichia coli*, thermal tolerance, wheat flour, foodborne pathogens

Wheat flour and other types of cereal flours are characterized by their low water activity (a_w) and are generally regarded as microbiologically safe (1, 2). Traditionally, these products have not been linked to foodborne disease caused by bacterial pathogens because their growth is not supported by the low-moisture environment (3). Flour is not normally consumed raw, as it is a component of other final products that typically undergo thermal processing, such as frying, cooking, or baking, leading to the elimination of bacterial pathogens (4). However, in recent years the realization that all

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grains are raw agricultural products subject to contamination from soil, animal feces, weather, insects, diseased plants, and other factors has questioned the belief that raw cereal grains are low-risk foods (5, 6).

In 2004, it was reported that wheat flour collected from growers, farm bins, and elevators of the Northern Plains of the United States contained up to 1.6×10^8 CFU/g of bacteria (7). Despite the low a_w, pathogenic and spoilage microorganisms may survive for long periods of time in flour or other dry foods (3). In addition, the milling process has little effect in reducing microbial contamination. Tainted flour may cause cross contamination or pose a risk to consumers who often taste cookie dough or batter mixes before baking (4). Hence, it is critical to conduct studies aimed to assess flour safety to develop a fundamental knowledge about the presence and survival of related pathogens. The interest in knowing more about the ability of foodborne pathogens to survive in low-a_w foods and food ingredients has grown over the past decades due to the occurrence of several outbreaks associated with these foods (8).

Since the 1950s, *Salmonella* has been the main pathogen of concern in dry foods, including flour. Because this bacterium has been related to an increased number of outbreaks, its presence and decontamination have been extensively studied (9–11). In 2005, the FDA announced that *Salmonella* might be present in wheat flour, and therefore its derivatives, such as dry cake mix, should not be considered ready-to-eat (RTE) food (12). In recent years, several outbreaks caused by enterohemorrhagic *Escherichia coli* (EHEC) have suggested that *Salmonella* is not the only pathogen of concern in wheat flour (8).

EHEC is a group of pathogenic *E. coli* strains that emerged during the past 4 decades. They have been responsible for many foodborne outbreaks worldwide (13). This diverse group includes a broad range of O:H serovars, among which O26, O45, O103, O111, O121, O145, and O157 have greater pathogenic potential and are isolated from foodborne disease patients at a higher frequency than others (14). There is very limited information about the presence of EHEC in wheat flour because almost no disease from this source had been recorded (2, 3, 15, 16). Previous studies reported only the presence of *E. coli* without identifying serotypes. The first reported outbreak of EHEC associated with wheat flour in the United States was in 2009, when a prepacked, ready-to-bake cookie dough was implicated as the vehicle of transmission. In that outbreak, *E. coli* O157:H7 caused 77 illnesses, 35 hospitalizations and 10 hemolytic uremic syndrome (HUS) cases (8).

In 2016, the first documented outbreak related to wheat flour and due to EHEC O26 and O121 was recorded (17). A multistate outbreak associated with raw flour consumption involved 63 people from 24 states and triggered a large food recall in the United States (18). In 2017, two smaller outbreaks of bloody diarrhea were reported in Canada, linked to different brands of wheat flour and caused by EHEC O121 (19). These epidemiology clusters also triggered large food recalls. The number of reports has continued to increase as pie and tart shells in Canada were involved in the most recent food report (20). These incidents clearly stress the need to reevaluate the safety of flour and flour-containing foods.

Because EHEC can pose a serious public health concern in flour, it is critical to conduct research to better assess the risk and develop interventions such as heat treatment to mitigate microbial loads. While the survival and thermal inactivation of EHEC in meat products have been extensively studied (22, 23), there is almost no information available for wheat flour or other low-a_w foods. Pathogen survival studies contribute to assessing the likelihood of survival under storage conditions. Microbial death kinetics are typically determined by linear and Weibull mathematical models (21, 24), which are instrumental in predicting the potential for viability of reduction by thermal treatments. The Weibull model has been used to effectively describe the inactivation of *Salmonella* in a number of low-a_w foods, including wheat flour (21).

This study was undertaken to assess the long-term survival of EHEC O26, O103, O111, and O157, four of the major EHEC serogroups to date in wheat, and to determine

the thermal death kinetics parameters of the same EHEC serogroups with appropriate microbial inactivation models.

RESULTS AND DISCUSSION

Wheat flour and inoculation procedure. All wheat flour samples used for serogroup cocktail inoculation had an initial background aerobic plate count of 2 log CFU/g or less. Research has shown that product composition and serotype may affect the rate of inactivation (21). Therefore, in this study we used a cocktail of 5 different EHEC strains of each serogroup to better account for the possible natural variability among strains (25). The inoculation method may influence the thermal resistance and survival of inoculated bacteria in challenge food studies (26). Liquid inoculation was reported to result in instability of *Salmonella* during equilibration, which can be expected since desiccation is an environmental stress adversely affecting viable bacterial numbers (27). The need for equilibration for several days and dough formation are some other characteristics of direct liquid inoculation that should be considered for wheat flour.

On the other hand, with dry inoculation it could be hard to get an inoculum level as high as 8 log CFU/g, and it may also cause variabilities (28, 29). In this study, a concentrated cell suspension method was effective to deliver homogeneous counts. The pre- and postinoculation a_w values of all flour samples used for long-term survival were 0.44 $\pm$ 0.05 and 0.47 $\pm$ 0.04, respectively, which were equivalent to a_w differences of less than 0.04 as a result of inoculation. The pre- and postinoculation a_w values in the case of flour samples used for thermal inactivation tests were 0.48 $\pm$ 0.069 and 0.57 $\pm$ 0.09, respectively, resulting in an a_w difference of less than 0.09.

The change in a_w of flour samples during long-term storage was relatively small (0.024 to 0.041). Measurements before and after the thermal treatments at their longest time point resulted in a change in a_w of less than 0.056 $\pm$ 0.024 regardless of treatment temperature. This is important because it is critical to keep the food sample within the designated a_w range, as a_w will affect the long-term survival and resistance of bacterial cells upon treatment (28, 30). Given the consistency of initial viable counts, the sequential mixing method using combined hand mixing and stomaching delivered homogeneously inoculated flour samples and an absence of bacterial cell clumps. Cell clumps, if present, may increase bacterial tolerance to antimicrobial interventions such as thermal treatment and may also result in variability of bacterial numbers (31).

Long-term survival of EHEC in wheat flour. Since a relatively high a_w is essential for microbial growth, one of the main strategies to control bacterial proliferation in food matrices is the reduction of a_w, which results in inhibition of enzymatic reactions and reduced metabolism (32). Surprisingly, a_w reductions seem to also enable bacteria to survive longer. Several researchers have reported that reduced a_w had a protective effect on bacteria against inactivation, and even some *Enterobacteriaceae*, such as *Salmonella*, survived for a long time in low-a_w foods, such as flour (33). Previous findings were consistent with the results of the present study.

Viable counts of EHEC O26, O103, O111, and O157 were detected in wheat flour stored at room temperature (23 $\pm$ 1°C) for up to 280 days (Fig. 1). The fastest reductions in viable EHEC counts were observed in the early postinoculation days. Interestingly, all serogroups had similar survival counts with multiple overlapping time points within the time course curves. The standard deviation of the counts across six subsamples (1 g each) of each serogroup in each measurement was $\leq$0.3 log CFU/g, which confirmed the homogeneous inoculum mixing of the flour samples.

There were approximately 1-log CFU/g reductions of the initial viable counts (approximately 8 log CFU/g) after the first 4 days, followed by approximately 0.5-log CFU/g declines up to day 10 of storage. For the following 4 weeks, the reduction rates lessened to approximately 0.5 log CFU/g per week and then to approximately 0.3 log CFU/g per week until 84 days of storage, which was the last time point at which surviving EHEC could be quantified by direct plating. After 16 weeks (112 days), all EHEC serovars were no longer quantifiable at 2 log CFU/g or greater (limit of quantification)

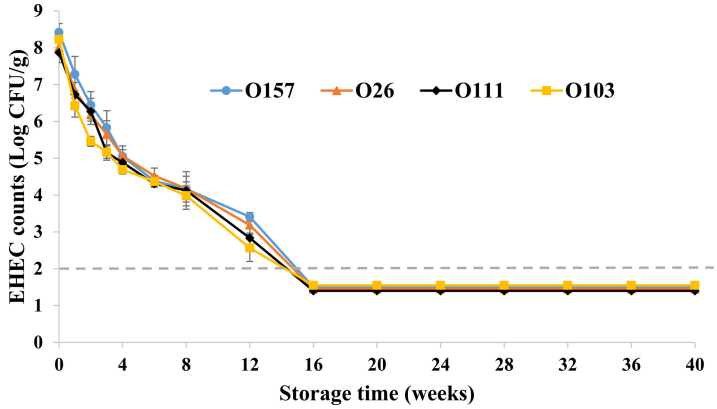

FIG 1 Long-term survival of enterohemorrhagic *Escherichia coli* serogroups O157, O26, O111, and O103 in wheat flour at room temperature (23 ± 1°C) for 36 weeks. Time points shown below the detection limit (dashed line) by direct plating (2 log CFU/g) indicate positive samples after enrichment. Samples from week 16 and after were enriched. Values are the log-transformed number of surviving cells per gram of sample, shown as the means from at least three independent trials with a minimum of two replicates with standard errors of the means indicated.

and were detected only by enrichment from week 16 up to week 40 (280 days) after 24 h of enrichment. EHEC O157:H7 was still detected 1 year after inoculation.

There is very limited information available on *Salmonella*'s long-term survival in wheat flour, but its long-term survival in peanut flour (34) stored at 20°C and in pasta (35) and raw pecan and peanut kernels (36) stored at room temperature for at least 12 months has been observed. Beuchat and Mann indicated that *Salmonella* survived in cookie and cracker sandwiches stored at 25°C for at least 182 days (37). In terms of *E. coli*, Baylis et al. (38) reported survival for 113 days for *E. coli* O157, O111, and O26 in mallow stored at 22°C at an a_w of 0.73 and an inoculum level of 4 log CFU/g. In the same study, survival durations were only 90 and 42 days for chocolate (a_w, 0.40) and biscuit cream (a_w, 0.75), respectively. These differences suggested an effect of matrix type on survival.

E. coli O157:H7 has also been reported to survive on walnut kernels (a_w, 0.45) at 23°C (39) and peanut and pecan kernels at 22°C (36) for at least 1 year. Interestingly, in most cases the counts from pecans were higher than from peanuts. Our results, in agreement with reports mentioned above, support the need for inclusion of EHEC survival data in hazard assessment of low-a_w foods, including wheat flour.

EHEC serogroups O26 and O157 in wheat flour were also stored at 35°C, and the viability was determined for 7 weeks. Similar to what was seen in the experiments at room temperature (23 ± 1°C), the inactivation curves of the two serogroups were almost identical (Fig. 2). The surviving EHEC numbers declined rapidly by approximately 2 log CFU/g in 3 days in contrast to only 1 log CFU/g in 4 days at 23°C. After only a week of storage, EHEC could not be detected without enrichment. Both serogroup samples required enrichment starting at week 2 of storage. However, while the last positive samples for serogroup O26 upon enrichment were observed after 28 days of storage, serogroup O157-positive samples were observed for up to 49 days.

The considerably higher reduction rate at 35 than at 23°C can be explained, as we know that survival of bacteria is favored by not only reduced a_w but also temperature (40). Previous works have indicated that bacterial survival in low-a_w foods is reduced as a result of increases in storage temperature (28, 41, 42). Also, *E. coli* was reported to survive in chocolate (a_w, 0.40) for 366, 90, and 43 days at 10, 22, and 38°C, respectively (38). In another study, survival of *Salmonella* on pecans stored at different temperatures up to 32 weeks was inversely correlated to the storage temperature (43).

Little is known about the physiology of pathogens and their mechanisms for survival and persistence in low-a_w foods in general (44), and investigating the mechanisms involved in desiccation tolerance is a relatively new area of research. To date, strategies

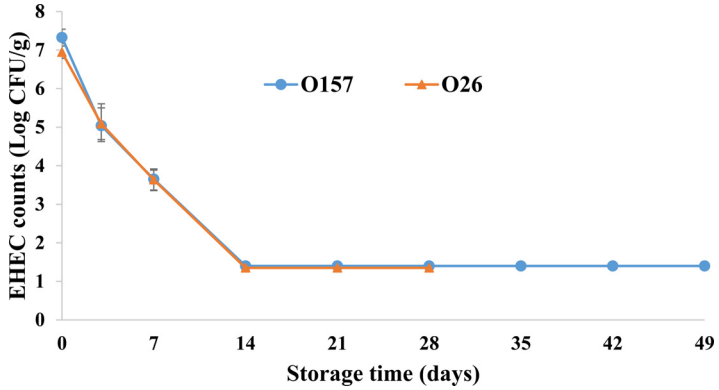

FIG 2 Survival of enterohemorrhagic *Escherichia coli* serogroups O157 and O26 in wheat flour at 35°C for up to 49 days. Time points shown below the detection limit (dashed line) by direct plating (2 log CFU/g) indicate positive samples after enrichment. Values are the log-transformed numbers of surviving cells per gram of sample, shown as the means from at least three independent trials with a minimum of two replicates with standard errors of the means indicated.

known to be used by the bacterial cells may include but may not be exclusive to filamentation of cells, accumulation of osmoprotectant metabolites, and switching to a metabolically dormant state (45), which might be negatively influenced by the increase in temperature, describing higher reduction rates for the microbial counts of flour stored at 35°C than at 23°C.

The stronger adverse effect of temperature increase on survival in wheat flour than in chocolate might be due to its lower fat content (34). Hiramatsu et al. also reported that all five strains each of *Salmonella*, *E. coli* O157, and *E. coli* O26 died after 35 to 70 days of storage at 35°C in desiccated paper disks (46), which is in agreement with our results. Hence, exposure of flour at 35 to 40°C for an appropriate length of time prior to distribution might be a feasible strategy for the milling industry to mitigate microbial risk for consumers.

Long-term inactivation kinetics of EHEC in wheat flour. The inactivation kinetics of EHEC in wheat flour stored at room temperature (23 ± 1°C) were calculated over the course of 84 days (Table 1). The decimal reduction times (*D* values) calculated with the log-linear first-order kinetics model of the four serogroups' inactivation ranged from 7.50 to 8.21 days, and their differences were not statistically significant ($P \leq 0.05$). The adjusted regression coefficients (R^2) values for all log-linear model regressions of long-term storage were greater than 0.83. Analysis of data obtained with the Weibull model showed first decimal reduction times (δ) of serogroups O26, O111, and O157 of 5.25, 5.31, and 5.07 days, respectively. The δ value measured for serogroup O103 was only 3.1 days, but none of the observed values were significantly different ($P \leq 0.05$). Survival curves from all serogroups had a similar concave shape (β value < 1). In addition, the adjusted R^2 values were consistently higher than in the log-linear model and greater than 0.96.

Long-term inactivation kinetics of EHEC in flour were consistent among serogroups. These results were comparable to the long-term survival counts, although R^2 values were

TABLE 1 Rates of enterohemorrhagic *E. coli* inactivation in wheat flour stored at room temperature (23 ± 1°C) for 84 days (12 weeks) calculated by linear and Weibull models[a]

	Linear model parameters			Weibull model parameters				
Serogroup	*D* (days)	*D* (SE)	Adjusted R^2	δ (days)	δ (SE)	β	β (SE)	Adjusted R^2
O26	8.21[a]	0.44	0.89	5.25[a]	1.74	0.55	0.04	0.98
O103	7.50[a]	0.14	0.84	3.10[a]	0.36	0.51	0.02	0.96
O111	7.99[a]	0.15	0.89	5.31[a]	0.77	0.57	0.02	0.96
O157	8.21[a]	0.35	0.83	5.07[a]	0.10	0.56	0.02	0.97

[a]Within each column, values followed by the same lowercase letter are not significantly different ($P \leq 0.05$).

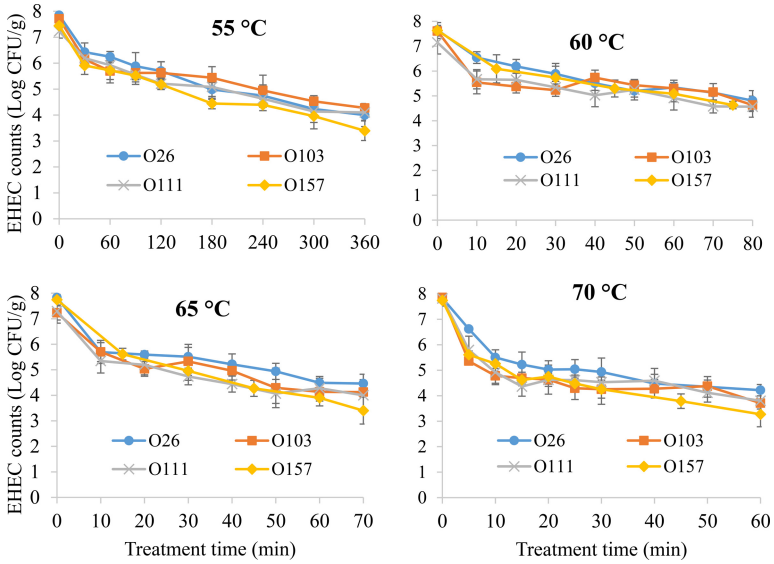

FIG 3 Thermal inactivation curves of enterohemorrhagic *Escherichia coli* serogroups O26, O103, O111, and O157 in wheat flour at 55, 60, 65, and 70°C. Values are the log-transformed numbers of surviving cells per gram of sample, shown as the means from at least three independent trials with a minimum of two replicates with standard errors of the means indicated.

above 0.83 and 0.96 for the log-linear and Weibull models, respectively, confirming the superiority of the Weibull model in terms of precision. This observation was similar to those of previous reports concluding that the Weibull model was a more appropriate tool for the death kinetics study of bacteria in low-a_w foods and a better basis to develop antimicrobial thermal interventions than the log-linear model (9, 21, 47).

Thermal inactivation kinetics of EHEC in wheat flour. When inoculated flour samples were heat treated at 55, 60, 65, and 70°C, the viable counts of all serovars decreased rapidly (Fig. 3). The inactivation curves of the four serovars were very similar at each particular temperature. Heating of flour samples at 55, 60, 65, and 70°C for 60 min resulted in average microbial count reductions of 1.66, 2.34, 3.32, and 4.06 log CFU/g, respectively.

Thermal inactivation kinetics parameters of EHEC serovars in wheat flour at 55, 60, 65, and 70°C were obtained by log-linear and Weibull models (Table 2). Similar to the

TABLE 2 Thermal inactivation rates of enterohemorrhagic *E. coli* in wheat flour calculated by Linear and Weibull models[a]

		Linear model parameters			Weibull model parameters					
Serogroup	Temp (°C)	*D* (min)	*D* (SE)	Adjusted R^2	δ (min)	δ (SE)	β	β (SE)	Adjusted R^2	Z (°C)
O26	55	$^{D}46.97^{a}$	1.46	0.86	$^{C}26.6^{a}$	3.32	0.51	0.03	0.97	12.64
	60	$^{C}11.24^{b}$	1.98	0.85	$^{B}9.24^{b}$	1.67	0.50	0.07	0.96	
	65	$^{B}9.74^{c}$	0.31	0.68	$^{AB}3.67^{c}$	0.00	0.39	0.00	0.95	
	70	$^{A}5.75^{d}$	0.04	0.83	$^{A}1.74^{d}$	0.80	0.41	0.09	0.95	
O103	55	$^{B}61.10^{a}$	9.03	0.74	$^{B}39.66^{a}$	10.68	0.46	0.03	0.90	6.7
	60	$^{A}11.40^{b}$	1.73	0.75	$^{A}5.07^{b}$	1.15	0.33	0.06	0.89	
	65	$^{A}9.34^{c}$	2.24	0.83	$^{A}3.53^{c}$	0.16	0.46	0.03	0.97	
	70	$^{A}8.67^{f}$	0.42	0.45	$^{A}0.14^{d}$	0.03	0.20	0.00	0.97	
O111	55	$^{B}52.03^{a}$	7.81	0.87	$^{B}37.25^{a}$	16.03	0.51	0.12	0.98	10.17
	60	$^{A}16.10^{b}$	1.65	0.77	$^{A}5.3^{b}$	2.52	0.35	0.07	0.96	
	65	$^{A}12.20^{c}$	0.17	0.61	$^{A}2.96^{c}$	0.29	0.32	0.03	0.93	
	70	$^{A}8.10^{ef}$	0.07	0.65	$^{A}1.04^{d}$	0.34	0.32	0.03	0.96	
O157	55	$^{C}46.53^{a}$	1.87	0.83	$^{B}15.56^{a}$	3.30	0.41	0.02	0.96	13.44
	60	$^{B}12.15^{b}$	0.62	0.82	$^{A}6.38^{b}$	0.19	0.43	0.01	0.98	
	65	$^{A}8.01^{c}$	0.32	0.88	$^{A}3.95^{c}$	0.82	0.49	0.03	0.98	
	70	$^{A}6.69^{de}$	0.44	0.76	$^{A}1.03^{d}$	0.00	0.35	0.00	0.96	

[a]Values preceded by different uppercase letters within each serogroup are significantly different. Values for the same treatment temperature within each column followed by different lowercase letters are significantly different ($P \leq 0.05$).

calculations for the long-term survival experiments, the viable count data had regression values that followed the Weibull model better than the log-linear model. Using the Weibull model, adjusted R^2 values of >0.95 were calculated, with the exception of serotype O103 at 55 and 60°C (adjusted R^2 values of 0.89 and 0.90, respectively) and serotype O111 at 65°C (adjusted R^2 value of 0.93). In comparison, the adjusted R^2 values obtained with the log-linear model were in the range of 0.45 to 0.88. As expected, the D and δ values obtained upon thermal treatment decreased as the temperature increased. However, the extent of the decline was not always consistent, either in each serogroup or between the four different serogroups at each particular temperature.

The D values obtained using the log-linear model were not significantly different among the serogroups at each temperature except for the 70°C treatment, in which the inactivation data for serogroup O26 resulted in a D value of 5.75 min, which was significantly different from the D values of 8.1 and 6.69 min for serogroups O111 and O157, respectively ($P \leq 0.05$) (Table 2). At 70°C, serogroup O103 inactivation data also had a different D value of 8.67 min, but the adjusted R^2 value was only 0.45. Within each serogroup, the 5°C increments in treatment temperature always resulted in a substantial decline of D values. D values obtained at 55°C were consistently different from the rest of similar measurements at higher temperatures, but the differences among D values at higher temperatures were not consistently significant.

The δ values obtained from the Weibull model substantially decreased with each 5°C increase in the treatment temperature (Table 2). At 70°C, the δ values ranged from 0.14 min for serotype O103 to 1.74 min for serogroup O26. None of the δ values calculated at the same temperature were significantly different among the four serogroups. The δ-value differences among temperatures were occasionally significant ($P \leq 0.05$), except for the 55°C treatment group, which consistently had δ values different from those of other temperatures. The change in temperature required to cause a 1-log reduction in δ value (Z value) was calculated with a log-linear regression with the four temperatures for each serotype. The regression coefficients (R^2) for the Z-value calculation were above 0.95 for serogroups O26, O111, and O157. The Z values were 12.6, 10.2, and 13.4°C for serogroups O26, O111, and O157, respectively, and they were not statistically significant. The Z value of serogroup O103 was 6.7°C ($R^2 = 0.91$), and this value was different ($P < 0.05$) from the serogroup O157's Z value.

Smith et al. reported D values of 9.97, 5.51, and 2.11 min at 75, 80, and 85°C for *Salmonella enterica* Enteritidis PT 30 in wheat flour (a_w, 0.43). They also reported δ values of 8.08, 4.97, and 1.59 min for the same temperatures (48). In another study, a D_{80} value of 6.9 min for *Salmonella* in all-purpose wheat flour with a_w of 0.45 was reported (49). In the present study, the EHEC's D, δ, and Z values were smaller than those previously reported for *Salmonella*, but they were still considerably higher than those for *E. coli* in many other types of matrices, including foods with higher a_w. A closed system using 0.5-ml PCR tubes was applied for thermal treatment procedures preventing changes in a_w decrease during the process, which may inversely affect the thermal death kinetics calculations (48, 49).

The only study on the thermal death kinetics of *E. coli* in wheat flour, to the best of our knowledge, reported a 5-log reduction for *E. coli* O157:H7 after 30 min at 70°C (50). Such a reduction rate is much higher than our findings, as 3-log CFU/g reductions were determined after 30 min at 70°C. This may partially be attributed to factors including but not limited to the inoculation and homogenization method used in the study, sampling method, thermal treatment procedure, and most importantly, the performance of thermal treatment immediately after the inoculation in that study. Shiga toxin-producing *E. coli* strains were reported to have D values in the range of 13.5 to 32.6, 0.6 to 1.2, and 0.05 to 0.24 min at 54.4, 60, and 65°C in wafers of ground beef (18). In our study, the D values for almost the same temperatures were in the range of 46.5 to 61.1, 11.2 to 16.1, and 8.0 to 12.2, respectively. This comparison strongly supports the idea that the thermal tolerance of EHEC in a low-a_w/low-fat matrix such as wheat flour increases as the water activity declines. In another study, *E. coli* O157:H7 had a Z value of 4.94°C in ground pork, whereas in this study the average Z value obtained was 10.73°C (51).

The shape of thermal death curves indicated that microbial reduction rates decreased as time passed regardless of temperature. This phenomenon, in addition to the fact that the Weibull model assumes that cells in the population have different degrees of resistance and a survival curve represents the cumulative form of a distribution of lethal events, might be the reason for the Weibull model's superiority for studying EHEC thermal death kinetics in flour. In contrast, first-order kinetics such as the log-linear model assume that each microorganism has the same probability of dying (9).

In conclusion, our findings demonstrate that EHEC can survive for 9 months in wheat flour stored under conditions typically found in commercial and home settings. Heat treatment of flour is a feasible strategy for the industry to mitigate microbial contamination, especially treatment at the temperatures used in this study, which are in a range that may have no or minimal influence on the wheat flour properties (functional, physicochemical, particle size, etc.) or may occasionally improve them (52–54). For wheat flour that cannot be heat treated, such as for specific cakes, the substitute antimicrobial intervention would be storing the final product at slightly high temperatures (35 to 40°C) for 2 months before distribution. This would effectively increase the microbial safety of flour, since *Salmonella* and EHEC will be inactivated as well as other pathogenic bacteria. Results from this study are useful for risk assessment and management for processors, providing sufficient fundamental knowledge for predicting the process lethality of EHEC in flour and for designing strategies to mitigate EHEC presence in the final product in order to prevent future infections.

MATERIALS AND METHODS

Bacteria and growth conditions. Twenty enterohemorrhagic *E. coli* strains were used in this research, which included five strains of each EHEC serogroup: O26 (3012–03, 3337–99, 3396–02, 3235–99, 3013–03), O103 (3002–98, 3009–02, 3284–02, 3316–02, 3425–01), O111 (3077–99, 3107–02, 3162–97, 3255–02, 3331–00), and O157 (ATCC 43895, 121583, E0018, 932, LC–40). All strains were obtained from the bacterial culture collection at the Center for Food Safety, University of Georgia, Griffin Campus. The strains from each serogroup were prepared as five-strain cocktails for inoculation. Stock cultures of individual strains were maintained at −80°C in tryptic soy broth (TSB; Difco Laboratories, Sparks, MD) supplemented with 20% (vol/vol) glycerol. Before use, all bacterial cultures were subjected to two consecutive transfers (24 h at 37°C). Working stocks of each strain were prepared using Luria-Bertani broth (LB; Difco Laboratories, Sparks, MD), stored at 4°C, and refreshed on a monthly basis. These working stocks were used to prepare the inoculation cultures.

Preparation of inoculums. Strains were individually inoculated into 40 ml TSB (Difco) and incubated for 24 h at 37°C with 200-rpm shaking. Cultures were centrifuged (3,000 × *g*, 15 min, 4°C) and individually resuspended in 10 ml sterile 0.1% buffered peptone water (BPW). The individual BPW strain suspensions (10 ml) were transferred into tubes and mixed to obtain two 25-ml suspensions of a 5-strain cocktail. Strain cocktail suspensions were centrifuged (3,000 × *g*, 10 min, 4°C), supernatants were removed, and the remaining pellets were harvested in 200 μl 0.1% BPW. An additional short spin and/or an additional 50 μl BPW was used if necessary for maximum pellet recovery, occasionally. This concentrated semiliquid pellet was used for flour inoculation.

Samples and inoculation procedure. The commercial all-purpose wheat flour was purchased from a retail store in Griffin, GA. Enumeration of background microflora was performed by at least three random measurements of 1-g samples from each bag. Each sample was diluted in 9 ml of sterile 0.1% BPW (Neogen, Inc., East Lansing, MI), and appropriate 10-fold serial dilutions were spread plated on tryptic soy agar (TSA; Difco Laboratories, Sparks, MD). After incubation (24 h at 37°C), bacterial numbers were transformed to log CFU/gram, and flour samples with background flora numbers of ≤2 log CFU/g were used for EHEC bacterial inoculation. The preinoculation water activity (a_w) of flour was measured at room temperature (23.5 ± 0.2°C) prior to every inoculation using a water activity meter (Aqua Lab model 3TE; Decagon Devices, Pullman, WA).

The concentrated cell suspensions from each serogroup five-strain cocktail were aseptically spot inoculated into 15-g flour portions in a sterile stomacher bag (Whirl-Pak; Nasco, Janesville, WI) and hand mixed for 5 min or until no inoculation clumps were visible. Subsequently, another 135 g flour was added to these seeded flour samples and manually mixed for 3 min, followed by two sets of stomaching (Seward Stomacher; 400 Lab System, Norfolk, United Kingdom) for 3 min at 260 rpm and a final 3-min manual mix. This procedure resulted in consistent initial EHEC inoculum levels of approximately 8 log CFU/g. Following flour inoculation, a_w was immediately measured as mentioned above. These 150-g inoculated flour samples were used for the long-term survival assays. For the thermal inactivation studies, the exact same procedure was performed, except that 100 g (10 g inoculated + 90 g) of flour was inoculated, resulting in initial inoculum levels of approximately 8.5 log CFU/g.

Packaging and storage. For long-term survival experiments, stomacher bags containing inoculated samples were tightly closed and put inside a Ziploc brand vacuum sealer bag. The bags were then sealed without vacuuming. Duplicate sealed bags of all four serogroups samples were put in plastic containers with tightly closed caps and stored at room temperature (23 ± 1°C) or in an incubator

at 35°C (serogroups O26 and O157) to study the effects of storage temperature. Sampling intervals were at days 0, 3, 7, 14, 21, and 28, followed by biweekly sampling at days 42 and 56 and then every 4 weeks. Sampling continued until reaching the limit of detection (2 log CFU/g) by standard microbiological plating, and then samples were enriched before detection. Testing of a specific inoculated sample stopped after three consecutive negative results were obtained. All samples were resealed immediately after each sampling interval.

Thermal treatments. Thermal inactivation was performed at 55°C (for up to 360 min), 60°C (for up to 80 min), 65°C (for up to 70 min), and 70°C (for up to 60 min) in 0.5-ml thin-wall PCR tubes with flat caps (Axygen Biosciences, Union City, CA) using a digital dry bath (Fisher Scientific, Pittsburgh, PA) to obtain thermal death curves for EHEC O26, O103, O111, and O157. Thermal treatments at each particular temperature were performed on 1 day, beginning from day 3 postinoculation. Based on our preliminary studies (data not shown) for each target temperature, the dry bath reading was set two degrees higher, resulting in the exact desired temperature inside the tubes. Temperature inside tubes was verified using a thermocouple bead wire temperature probe attached to a portable thermometer (HH series; Omega Engineering Inc., Stamford, CT). Inoculated flour samples (0.33 ± 0.02 g) with each five-strain serogroup cocktail were treated at once with same-time intervals for each specific temperature. Once removed from the thermal block, the tubes were immediately placed in an ice-water bath for 1 min to stop inactivation before they were subjected to microbial analysis.

Microbiological analysis. For storage, duplicate 150-g flour samples were sampled in triplicate 1-g portions at each sampling interval followed by 10-fold serial dilution in 0.1% BPW (Neogen, Inc., East Lansing, MI), spread plated on TSA, and incubated at 37°C for 24 h. Following incubation, colonies were counted and transformed into log CFU/gram for further analysis. After reaching the limit of detection without enrichment, 1-g samples from each stored bag were transferred to 40 ml of lauryl tryptose broth (LTB; Difco) and enriched for 24 h at 37°C with 200-rpm shaking.

Upon enrichment, appropriate dilutions of the preenriched homogenates were spread plated on sorbitol MacConkey agar (SMAC; Difco) supplemented with 0.05 mg/liter cefixime and 2.5 mg/liter potassium tellurite (Sigma-Aldrich, Inc., St. Louis, MO) (CT-SMAC) (55) or loaded onto 3M Petrifilm *E. coli*/coliform count plates (6404; 3M Microbiology, St Paul, MN) according to the manufacturer's guidelines and incubated at 37°C for 24 h. A random confirmation step was performed on all TSA and CT-SMAC presumptive EHEC colonies using a serogroup-specific agglutination test (Cedarlane, Burlington, Canada). In the case of thermally treated samples, each tube's content was transferred to 9.7 ml 0.1% BPW and appropriate 10-fold serial dilutions were spread plated on TSA for microbial enumeration. Colony counts were transformed to log CFU/gram accordingly and used for data handling and statistical analysis.

Data handling and statistics. Survival counts were calculated using the Food and Drug Administration's *Bacteriological Analytical Manual*'s formula for aerobic plate count modified for 0.1-ml plating volumes (22). All data points were the results of averaging triplicate measurements at each sampling time. A minimum of two replicate trials were used for every test ($n = 6$).

Data obtained were fit to the log-linear model (23) and Weibull model (56) using Microsoft Excel 2016 Add-in GinaFit version 1.7 (57).

The log-linear model (23) uses the following equation:

$$N_t = N_0 \cdot e^{(-k_{\max} \cdot t)}$$

where N_t is the population at time t (CFU/gram), N_0 is the population at time zero (CFU/gram), and $k_{\max}$ is the maximum specific inactivation rate (minute^{-1}).

The Weibull model (56) uses the following equation:

$$\log\ (N_t) = \log\ (N_0) - \left(\frac{t}{\delta}\right)^{\beta}$$

where N_t and N_0 are as previously described, δ is the time required for the first decimal reduction (minutes), and β is a fitting parameter that describes the shape of the curve (if β is >1, the curve is convex; if β is <1, it is concave).

The D values were calculated as $1/k_{\max}$ after fitting the data to the log-linear model. The averages of inactivation parameters were used for further analysis. The Z values (increase of temperature required to decrease the δ value by 1 log) were calculated by plotting the log of δ values into a linear regression scatter plot in Microsoft Excel 2016.

Means of inactivation parameters in survival counts (log CFU/gram) from long-term storage and thermal inactivation tests were subjected to analysis of variance (ANOVA). Tukey's multiple-range test was applied for the long-term survival and analysis of values obtained from the same temperature treatments. Fisher's least significant difference (LSD) test was applied for the comparison of different treatment temperatures in each serogroup. All analyses were performed using IBM SPSS Statistics version 24 (SPSS Institute, Chicago, IL). The significance of difference was defined at P values of ≤ 0.05.

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