Moisture content of bacterial cells determines thermal resistance of *Salmonella* Enteritidis PT 30

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Running title: *Salmonella* control by moisture content adjustment

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Abstract

Salmonella spp. are resilient bacterial pathogens in low-moisture foods. There has been a general lack of understanding of critical factors contributing to the enhanced thermal tolerance of *Salmonella* spp, in dry environments. In this study, we hypothesized that the moisture content (X_W) of bacterial cells is a critical intrinsic factor influencing the resistance of *Salmonella* spp. against thermal inactivation. We selected *Salmonella* Enteritidis PT 30 to test this hypothesis. We first produced viable freeze-dried *S.* Enteritidis PT 30, conditioned the bacterial cells to different X_W (7.7, 9.2, 12.4 and 15.7 g water/100g dry solids), and determined thermal inactivation kinetics of those cells at 80 ℃. The results show that *D*-value (time required to achieve one-log reduction) decreased exponentially with increasing X_W. We further measured water activities (a_w) of the freeze-dried *S.* Enteritidis PT 30 as influenced by temperature between 20 and 80 ℃. By using those data, we estimated the X_W of *S.* Enteritidis PT 30 from the published papers that related *D*-values of the same bacteria strain at 80 ℃ with a_w of five different food and silicon dioxide matrices. We discovered that the logarithmic *D*-values of *S.* Enteritidis PT 30 in all those matrices also decreased linearly with increasing X_W of the bacterial cells. The findings suggest that the amount of moisture in *S.* Enteritidis PT 30 is a determinant factor on their ability to resist thermal inactivation. Our results may help future research into fundamental mechanisms for thermal inactivation of bacterial pathogens in dry environments.

Keywords: moisture content, bacterial cells, thermal resistance, *Salmonella* Enteritidis PT 30, low-moisture foods

Importance

This paper established a logarithmic relationship between the thermal death time (D-value) of *S.* Enteritidis PT 30 and the moisture content (X_W) of the bacterial cells by conducting thermal inactivation tests on freeze-dried S. Enteritidis PT 30. We further verified this relationship using literature data for *S.* Enteritidis PT 30 in five low moisture matrices. The findings suggest that X_W of *S.* Enteritidis PT 30, which is rapidly adjusted by microenvironmental a_w, or relative humidity, during heat treatments, is the key intrinsic factor determining thermal resistance of the bacterium. The quantitative relationships reported in this study may help guide future designs of industrial thermal processes for control of *S.* Enteritidis PT 30 or other *Salmonella* stains in low-moisture foods. Our findings highlight a need for further fundamental investigation into the role of water in protein denaturation and accumulation of compatible solutes during thermal inactivation of bacterial pathogens in dry environments.

Introduction

Free water in foods is required to support the growth of yeasts, molds, and bacteria (1). Water activity (a_w) is an indirect measure of the free water available to the microorganism in food systems (2). Generally, low-moisture foods (LMFs) have an a_w at or below 0.6 at room temperature (3). The low a_w environment in low-moisture foods, such as milk powder, chocolate, peanut butter, and cereal, inhibits the growth of microorganisms (4, 5). However, pathogens like *Salmonella* can survive in low-moisture foods during storage and frequently cause worldwide outbreaks (1, 3, 6–8).

Salmonella spp. are one of the most common pathogens causing severe foodborne illness for humans (7, 8). These resilient microorganisms can easily adapt to environmental conditions beyond their optimal growth range, including temperature, pH and a_w (1, 5, 9). The minimum a_w for the growth of *Salmonella* is 0.94, but they can survive and remain viable in low-moisture foods for long periods of time (4). In particular, *Salmonella* has enhanced thermal resistance in dry conditions. Studies have shown that their thermal resistance, as evaluated by *D*-value (the time required to inactivate 90% of target bacteria at a fixed temperature), increases sharply with reducing a_w in various low-moisture foods (3, 5, 10–13).

Early researchers attempted to establish a direct correlation between thermal resistance of foodborne pathogens at certain treatment temperatures and the a_w of the inoculated foods at room temperature (10). Recent studies, however, have shown that the a_w of food systems at room temperature does not accurately reflect the microenvironment that foodborne pathogens are exposed to in a thermal treatment. For instance, when wheat flour, whey protein, and almond flour are conditioned to an a_w of 0.45 at room temperature and then heated to 80 °C in sealed containers (without moisture loss), their a_w changed to 0.68, 0.62, and 0.54, respectively (11). *D*-

values at 80 °C ($D_{80°C}$-values) of *S.* Enteritidis PT 30 in these foods were about 5, 11, and 21 min, respectively (11). Thus, the a_w of foods measured at room temperature should not be considered as a process control parameter in design of thermal inactivation processes. Our most recent studies have demonstrated that the a_w of food systems measured at treatment temperatures ($a_{w,\ treatment\ temperature}$) should be considered as a critical extrinsic factor determining the *D*-values of micro-organisms during thermal treatments (11, 13–16).

The a_w of a biomass is expressed as its water vapor pressure to the saturated water pressure at a given temperature. It is a thermodynamic parameter that generally changes with temperature in food systems (11, 15, 17–19). Thus, it is an extrinsic property of a biomaterial. In a high moisture environment, water accounts for 60% to 90% of the mass of bacterial cells (20). In low moisture foods, however, bacterial cells respond to the a_w of the food matrices in multiple ways, and a straightforward approach is the adjustment of moisture content (X_W, g water/100g dry solids,) of bacterial cells through moisture diffusion (17, 21). The moisture diffusion between bacterial cells and their surrounding food matrices in a thermal process is illustrated in Fig. 1. Even though the biomass of bacterial cells and the contaminated food have the same a_w at room temperature, their a_w may change when heated to a high temperature. The differences in a_w between bacterial cells and the food creates a moisture vapor pressure gradient that drives moisture diffusion in or out of the bacterial cells until an equilibrium is reached. In industrial operations, the food matrices have much larger masses than the bacteria that potentially contaminate the foods. Thus, the a_w of the bacterial cells should be equal to that of the foods at equilibrium. As shown in Fig. 1A, bacterial cells will gain moisture from their microenvironment when a_w of the food is higher than that of bacterial cells at thermal-treatment temperatures. On other hand, the bacterial cells would be dehydrated if a lower a_w is provided by a food system

(Fig. 1B). The moisture diffusion between bacterial cells and their food environment can be rapid (within seconds) because of the smaller characteristic size of bacterial cells (17). Therefore, we hypothesize that X_W of bacteria might be the dominant intrinsic parameter that determines their thermal resistance in low-moisture foods in a thermal process.

The relationship between X_W and a_w of a biomass at different temperatures, commonly referred to as the moisture sorption isotherms. Extensive data on the moisture sorption isotherms for foods exist in the literature. But no research has been published on the isotherms for bacteria until the work of Syamaladevi et al. (17) who reported the moisture sorption isotherms for a biomass of freeze-dried *Enterococcus faecium*, a commonly used surrogate of *Salmonella*. There has been no published research on a_w changes of the biomass of *Salmonella* cells with temperature.

The goal of this research was to test the hypothesis that moisture content of bacterial cells is the intrinsic parameter determining the thermal resistance of *Salmonella* in low moisture environments. The specific objectives of this study were to: 1) determine the thermal resistance of freeze-dried *Salmonella* Enteritidis PT 30 at 80 °C with different X_W (7.7, 9.2, 12.4 and 15.7 g water/100g dry solids), 2) establish the relationship between X_W and a_w of freeze-dried *S.* Enteritidis PT 30 biomass at different temperatures, and 3) study the correlation between X_W and thermal resistance of *S.* Enteritidis PT 30 in low-moisture matrices.

We selected *S.* Enteritidis PT 30 as the target bacterium in this study because its thermal resistance in different low moisture matrices have been reported (10, 12, 22–24). We used freeze-drying, also known as lyophilization, to produce desiccated *S.* Enteritidis PT 30 samples with a high vitality (22). The X_W of a porous mass of freeze-dried *S.* Enteritidis PT 30 could easily be adjusted by exposing it to different relative humidity. We conducted thermal

122 inactivation tests on freeze-dried *S.* Enteritidis PT 30 at different values of X_W using improved
123 thermal-death-time (TDT II) cells and investigated the relationship between $D_{80°C}$ values and X_W.
124 We also measured temperature-dependent changes in a_w of freeze-dried *S.* Enteritidis PT 30 cells
125 at different X_W to establish the relationships between X_W and a_w of *S.* Enteritidis PT 30 biomass at
126 difference temperatures (isotherms). Finally, we estimated the X_W of *S.* Enteritidis PT 30 in five
127 low-a_w systems reported in the literature, generated the linear regression for the logarithmic $D_{80°C}$
128 values against the X_W in these matrices, and compared it to that of freeze-dried *S.* Enteritidis PT
129 30.

130 ## Results and Discussion

131 ### Thermal inactivation kinetics of freeze-dried *S.* Enteritidis PT 30

132 The population of concentrated *S.* Enteritidis PT 30 inoculum was ~12 log CFU/mL. The
133 initial viability of freeze-dried *S.* Enteritidis PT 30 was 10.72 ± 0.34 log CFU/g, and less than 2
134 log reduction in population caused by freeze-drying. The samples were pre-equilibrated under
135 different relative humidity or equivalent a_w values at room temperature (~21 °C). In the thermal
136 inactivation tests, the come-up time (CUT, min) for the core temperature of freeze-dried *S.*
137 Enteritidis PT 30 samples to reach 79.5 °C was 1.5 min when heated to 80 °C in TDT II test cells.
138 The representative thermal inactivation curves for freeze-dried *S.* Enteritidis PT 30 having with
139 different X_W (7.7, 9.2, 12.4 and 15.7 g water/100g dry solids) are shown in Fig. 2. The population
140 reduction followed the typical first-order linear regression relationship with the treatment time
141 (Eq. 1), with a R^2 value between 0.84 and 0.98.

142 More rapid microbial reductions ($log\left(\frac{N}{N_0}\right)$) were observed at higher X_W as shown in Fig. 2,
143 which are consistent with the thermal inactivation trends of *Salmonella* and *Enterococcus*
144 faecium in several low-a_w systems (10, 11, 14, 25, 26).

145 **Changes in *D* value with X_W of bacterial cells**

146 Microbial survival from thermal inactivation were analyzed using log-linear model and
147 Weibull model (Eq. (1) & (2), Table 1). Given that the Weibull model yielded no significant
148 improvement in fitness, the log-linear model was used for subsequent analyses and comparison.
149 The $D_{80°C}$-values of freeze-dried *S.* Enteritidis PT 30, obtained from the slope of the trend lines in
150 Fig. 2, were 27.7 ± 0.7, 11.9 ± 0.2, 6.5 ± 1.1 and 1.9 ± 0.6 min at X_W of 7.7, 9.2, 12.4 and 15.7 g
151 water/100g dry solids, respectively (Table 1). The $D_{80°C}$-values as influenced by bacterial cell X_W
152 are shown in Fig. 3. In general, the $D_{80°C}$-values decreased exponentially with increasing X_W of
153 bacterial cells. This suggests that X_W of bacterial cells is a critical factor that determines the
154 thermal resistance of *Salmonella* in thermal treatments.

155 **Moisture sorption isotherms of freeze-dried *S.* Enteritidis PT 30**

156 The temperature dependent changes in a_w of freeze-dried *S.* Enteritidis PT 30 containing
157 different X_W as measured by high-temperature-cells and analyzed by Clausius-Clapeyron
158 equation (CCE, Eq. 3) are presented in Fig. 4. Generally, the a_w of freeze-dried *S.* Enteritidis PT
159 30 increased linearly with increasing temperature at a specific X_W. This trend is similar to that of
160 high-protein and high-starch food systems, including corn starch, soy protein and wheat flour (12,
161 18). However, it dramatically differs from that of high-oil and sugar-rich foods. Specifically, a_w
162 values of peanut oil, coconut milk powder and almond flour, do not increase and may even
163 decrease with increasing temperature (14, 16, 28,).

164 The effect of temperature on the a_w changes of freeze-dried *S.* Enteritidis PT 30 was more
165 obvious in the samples with a relatively lower X_W. For instance, at the X_W of 9.2 g water/100 g
166 dry solids, the a_w of freeze-dried *S.* Enteritidis PT 30 at 20 ℃ ($a_{w,20\ ℃}$) was 0.33. When heated to
167 80 ℃, a_w of freeze-dried *S.* Enteritidis PT 30 ($a_{w,\ 80℃}$) was increased to 0.52. The a_w increased by
168 0.19. When the X_W of the bacterial cells was increased to 22.3 g water/100 g dry solids, the a_w of
169 freeze-dried *S.* Enteritidis PT 30 increased from 0.83 to 0.94 as temperature was elevated from
170 20 ℃ to 80 ℃, where a smaller increment in a_w was obtained (0.11 < 0.19). Similar result has
171 also been reported in multiple low moisture food systems, including wheat flour, almond flour,
172 non-fat milk powder, corn starch, soy protein, cheddar cheese powder and coconut milk powder
173 (18, 19, 27).

174 The relationship between X_W of freeze-dried *S.* Enteritidis PT 30 biomass and their a_w at
175 different temperatures, commonly referred to as moisture sorption isotherms, is shown in Fig. 5.
176 The specific parameters for GAB equations (Eq. 4) at different temperatures in this study are
177 shown in Table 2. The derived monolayer moisture content (X_m) of freeze-dried *S.* Enteritidis PT
178 30 ranged from 10.0 g water/100 g dry solids at 20 ℃ to 7.5 g water/100 g dry solids at 80 ℃
179 (Table 2). These values are much larger than the X_m of most protein-rich systems. For example,
180 Perez-Reyes at al. (2020) recently reported X_m of 6 g water/100 g dry solids at 20 ℃ and 5 g
181 water/100 g dry solids at 80 ℃ for egg white powders (84.3% protein, 0.6% fat, d. b.), and 4 g
182 water/100 g dry solids at 20 ℃ and 3 g water/100 g dry solids for whole egg powder (46.4%
183 protein, 55.1% fat, d. b.) (28). This suggests that the biomass of freeze-dried *S.* Enteritidis PT 30
184 had much larger water binding capacity than those protein-rich powders.

185 In general, the X_W of freeze-dried *S.* Enteritidis PT 30 increased with increasing a_w at any
186 given temperature (Fig. 5). For example, the X_W of freeze-dried *S.* Enteritidis PT 30 was 9.2 g
187 water/100 g dry solids when conditioned to a_w of 0.3 at 20 ℃. It increased to 15.0 g water/100 g
188 dry solids when conditioned to a_w of 0.6 at the same temperature. The values of a_w of freeze-
189 dried *S.* Enteritidis PT 30 at fixed X_W increased significantly when heated from 20 ℃ to 80 ℃, as
190 shown in Fig. 5. The sorption isotherms of freeze-dried *S.* Enteritidis PT 30 resemble a part of
191 type II isotherm (S-shaped), exhibiting a sigmoid-shape curve. The two slightly bending areas
192 were observed at a_w below 0.3 and above 0.7. However, J-shaped isotherms were reported for
193 freeze-dried *E. faecium* cells (14). The difference might be attributed to the different cell wall
194 structures and compositions between Gram-positive and Gram-negative bacteria. *Salmonella* is a
195 Gram-negative bacterium; its cell wall consists of an outer membrane, a thin-layer peptidoglycan
196 and a cytoplasmic membrane. *E. faecium*, on the other hand, is a Gram-positive bacterium; its
197 cell wall consists of a thicker layer of peptidoglycan and one cytoplasmic membrane (29).

198 High-protein content or starch-rich food systems, such as corn starch, soy protein, and
199 wheat flour, usually show the same type of isotherm curves as freeze-dried *S.* Enteritidis PT 30
200 (6, 18). But, for high-sugar content and oil-rich food systems, the moisture sorption isotherms
201 generally show a type III isotherm (J-shaped), which is concave upward due to more moisture
202 gain from the surface crystalline dissolution at higher a_w (27). The a_w of peanut butter also
203 increased sharply with an extremely small increment of X_W at room temperature, and this
204 increase was more significant at higher treatment temperature (6).

205 **Net isosteric heat of sorption between freeze-dried *S.* Enteritidis PT 30**

206 The net isosteric heat of sorption (q_{st}), defined as the total enthalpy change for sorption
207 minus the specific latent heat of vaporization of liquid water (30), is a unique parameter for

208 different biomasses. The value of q_{st} reflects the bond energy between water molecules and solid
209 substances, which can be obtained from an empirical relation at a specific X_W (31, 32). In this
210 study, q_{st} was related to X_W (g water/100 g dry solids) of bacterial cells through an exponential
211 relation:

212 $$q_{st} = 17.852\exp(-0.10 \times X_W) \quad (5)$$

213 where the goodness of fit (R^2) was more than 0.99. A comparison of q_{st} for freeze-dried *S.*
214 Enteritidis PT 30 and several representative low-a_w systems is shown in Fig. 6.

215 As shown in Fig. 6, q_{st} correlates negatively with X_W. The higher q_{st} values obtained at
216 lower X_W indicate stronger bonds between water molecules and the solid matrices (33). That is,
217 more energy is required to break the bonds at lower X_W. The relationship between q_{st} and X_W of
218 freeze-dried *S.* Enteritidis PT 30 sharply differs from that of the foods shown in Fig. 6. The q_{st}
219 value of freeze-dried *S.* Enteritidis PT 30 is in general higher than that of the high protein and
220 high oil content foods at X_W between 6 to 10 g water/100 g dry solids and all the selected foods
221 when X_W is above 15 g water/100 g dry solids. This observation is consistent with the high values
222 of monolayer moisture contents, X_m, of freeze-dried *S.* Enteritidis PT 30 discussed earlier.

223 A sharp drop in q_{st} with increasing X_W was observed for all the foods shown in Fig. 6. The
224 deepest drop was obtained for oil-rich foods, such as coconut milk powder (consisting of 63.8%
225 fat, d.b.(18)) and almond flour (consisting of 50.7% fat, d.b.(11)), followed by intermediate
226 foods and protein-rich foods (non-fat milk powder and soy protein), and finally high-
227 carbohydrate foods, like organic wheat flour (86.1% carbohydrate, d.b.(11)) and corn starch
228 (98.0% carbohydrate, d.b. (18)). Lipids are hydrophobic. The hydrophobic interaction between
229 lipids and water is weaker than hydrogen bonds, resulting in a decreased enthalpic demand to

break the bonds between water molecules and the solids (34). Carbohydrates are hydrophilic macromolecules, and polysaccharides (such as starch) have strong affinity to water molecules because of multi-hydroxyl (-OH) groups (35). Combined interactions of hydrogen bonds and glycosidic bonds in carbohydrates are energetically favorable, leading to large amounts of energy required to break the bonds between water and the solids. Thus, high-carbohydrate foods such as corn starch have the highest q_{st} when compared to other foods at a specific X_W. Proteins are polymers with complex structures, and multiple bonds are present, including peptide bonds (primary structure), hydrogen bonds (secondary and tertiary structure), ionic bonds and disulfide bonds (tertiary structure) (36). Proteins have less affinity to water than polysaccharides, so the q_{st} of protein-rich foods is smaller than that of carbohydrate-rich foods.

Bacterial cells consist of more complex chemical compounds, including proteins, RNA, phospholipids and polysaccharides (37). The chemical bonds with water molecules in bacterial cells are also complex, because the cells may have unique capacities to retain moisture when compared to the food matrices included in Fig. 6. Although it is difficult to directly connect the $D_{80°C}$-values with q_{st} of freeze-dried *S.* Enteritidis PT 30, it is interesting to note that they both decrease exponentially with the X_W of the bacterial cells (Fig. 3 and 6).

Comparison of Log *D*-values against X_W of *S.* Enteritidis PT 30 in different matrices

Log $D_{80°C}$-values of *S.* Enteritidis PT 30 in silicon dioxide, wheat flour, whey proteins, honey powder, and almond flour, are summarized in Table 3 (6, 10, 11, 38). In those studies, the liquid *S.* Enteritidis PT 30 inoculum was inoculated into the mentioned matrices (wet inoculation), and then conditioned for 3-5 days to different at room temperature. *D*-values of the bacterium at 80 °C were reported against a_w of the matrices measured at the treatment temperature, 80 °C (see Table 3). We estimated the X_W of *S.* Enteritidis PT 30 in these low-a_w

253 systems using GAB model (Eq. 4), and the values are included Table 3. With those data, we
254 developed a linear regression line between the Log $D_{80°C}$-values and the estimated X_W for S.
255 Enteritidis PT 30 in the low-a_w systems (red dashed line, R^2=0.88), as shown in Fig. 7. The linear
256 regression for Log $D_{80°C}$-values of freeze-dried *S.* Enteritidis PT 30 against its X_W obtained from
257 this study is also plotted as the dark solid line (R^2=0.98) in Fig. 7, along with 95% confidence
258 intervals represented by the grey shadow area. Interestingly, the Log $D_{80°C}$-values of *S.*
259 Enteritidis PT 30 in silicon dioxide and different low-moisture foods are scattered mostly within
260 the 95% confidence interval area for freeze-dried *S.* Enteritidis PT 30. The regression line
261 derived from the reported data in low-a_w systems is found to entirely fall within the 95%
262 confidence intervals. Moreover, the regression lines for freeze-dried *S.* Enteritidis PT 30 and
263 low-a_w systems are almost overlapped. This suggests that regardless of the matrices, the thermal
264 resistance of *S.* Enteritidis PT 30 was largely determined by its X_W. This is reasonable, because
265 a_w of the microenvironment uniquely controlled the X_W of the bacterial cells according to the
266 relationships in Fig. 5, regardless of the matrices.

267 **Interpretation of thermal resistance of *Salmonella* in low-a_w systems**.

268 The a_w directly reflects the moisture vapor pressure within the food systems where bacteria
269 are imbedded. As demonstrated in Fig. 1, moisture diffusion occurs between the bacterial cells
270 and the microenvironment when their a_w values are not the same during heating. In general, the
271 trends of a_w changes in low-moisture foods with high-protein or starch content are similar to that
272 of bacterial cells when heated to a high temperature (11, 17–19), and the X_W of the bacterial cells
273 will not change much during a thermal treatment. However, the a_w of oil-rich foods is stable or
274 even decreased when heated to high temperatures (11, 18, 19). The bacterial cells in oil and oil-
275 rich foods may be dehydrated during thermal treatments (15). For instance, when the *S.*

Enteritidis PT 30 imbedded wheat flour (protein and starch-rich) or almond flour (oil-rich) are equilibrated to a_w of 0.45 at room temperature and then heated to 80°C, the a_w of freeze-dried *S.* Enteritidis PT 30 would increase to 0.65 as predicted by CCE (Eq. 3) of this study. The $a_{w,\ 80°C}$ of wheat flour and almond flour would increase to 0.68 and 0.54, respectively (Table 4). According to the GAB modeled moisture sorption isotherms at 80 °C (Table 2), the X_W of freeze-dried *S.* Enteritidis PT 30 at $a_{w,\ 80°C}$ of 0.65 would be 12.2 g water/100 g dry solids. Due to the moisture diffusion, the X_W of the bacterial cells would increase to 12.8 g water/ 100 g dry solids (gaining 0.6 g water/100 g dry solids) in wheat flour, but decrease to 10.0 g water/ 100 g dry solids in almond flour (losing 2.2 g water/100 g dry solids, Table 4). According to the exponential relation in Fig. 3, the $D_{80°C}$ of freeze-dried *S.* Enteritidis PT 30 in the wheat flour would be 5.9 min when X_W of the bacterial cells is 12.2 g water/100 g dry solids , which is slightly higher than the reported $D_{80°C}$ of *S.* Enteritidis PT 30 in wheat flour (4.9 min)(11). In contrast, the $D_{80°C}$-values of *S.* Enteritidis PT 30 in almond flour would be 21.2 min, almost 5 times higher than that in wheat flour. Similar observations can be made when two foods are pre-conditioned to an a_w of 0.6 at room temperature and heated to 80 °C (as summarized in Table 4). It is shown from the above examples that the equilibrium X_W of *S.* Enteritidis PT 30 in heated food matrices is the dominant factor determining their resistance in thermal treatments.

The results of this study allow prediction the X_W of *S.* Enteritidis PT 30 in a low-moisture food by using the GAB Model (Eq. 4 and Table 2) and make it possible to estimate the corresponding value of *D*-value of *S.* Enteritidis PT 30 by using the exponential equation in Fig. 3. Take peanut oil as an example, if the a_w of peanut oil is 0.53 at room temperature, it will reduce to 0.21 when heated to 80 °C according to Yang et al. (39). The estimated X_W of *S.* Enteritidis PT 30 in the peanut oil will be 4.8 g water/100g dry solids and the corresponding $D_{80°C}$

299 will be 59.4 min. That is, it would take 6-hour heating at 80 °C to achieve a 6-log reduction of *S.*
300 Enteritidis PT 30 in peanut oil with an initial a_w of 0.53.

301 It is reasonable to speculate that the thermal inactivation of *S.* Enteritidis PT 30 is mainly
302 caused by irreversible stereochemical structural alterations of the hydrophilic protein
303 components of the cells at 80 °C (40). Aggregation of cytoplasmic proteins (including ribosomes)
304 and denaturation of DNA in high moisture *S.* Enteritidis PT 30 cells were observed from
305 transmission electron micrographs (TEM) after 10-min thermal treatment at 80 °C; whilst no
306 visible aggregates were found in dried cells within 60 min heating at 80 °C (40). Indeed, it has
307 been reported that the thermally destroyed bacterial cells with high X_W is mainly attributed to the
308 loss of functionality of proteins (40–42). Cytoplasmic proteins, DNA, and ribosomal RNA of
309 bacterial cells are considered to be the major cellular targets in microbial inactivation induced by
310 heat (43). It has also been reported that the thermal denaturation of proteins is more effective in
311 water than in dry air due to the preferential hydration of solutes in low moisture conditions
312 providing protection against protein denaturation (29, 44, 45). Dehydration of proteins would
313 induce conformational transitions (46), the absence of water may prevent proteins from
314 deformation (47, 48) and produce more compatible solutes to counteract the environmental stress
315 (45), and thus, may somehow stabilize aggregated-structured cytoplasmic proteins, DNA and
316 ribosomal units against thermal-induced damages (40, 48). More systematic studies are needed to
317 study the roles of water molecules in structural alterations of key cellular components, in
318 particular on the rate of denaturation of functional proteins, during thermal inactivation of
319 microbial organisms.

320 **Conclusion**

This study established a quantitative relationship between X_W and D-values of freeze-dried *S.* Enteritidis PT 30. The moisture sorption isotherms provided the bridge to compare our results with previously reported data on thermal resistance of *S.* Enteritidis PT 30 in multiple low-a_w matrices. The linear relationship between Log D-values of *S.* Enteritidis PT 30 and its X_W can be used to predict its thermal resistant in different low-moisture foods and design effective industrial thermal processes for control of *Salmonella* in low-moisture foods. Our study provided experimental evidence to support the hypothesis that the thermal resistance of bacteria is intrinsically determined by the amount of moisture in the bacterial cells which is adjusted by the a_w of low-moisture foods or environmental relative humidity at the treatment temperatures. Food systems, such as wheat flour, that have large increases in a_w during heating will cause hydration of bacterial cells (increase in cell X_W), making them more vulnerable to thermal treatments. On the other hand, bacterial cells in oil-rich foods are more difficult to be inactivated because of the desiccation. Similarly, in an open environment, bacterial cells are more difficult to be inactivated in low relative humidity environments than at high relative humidity. This work also strengthens the importance of a_w at thermal treatment temperature of contaminated food in predicting the inactivation of pathogens. However, this study was limited to *S.* Enteritidis PT 30. It is of interest to explore direct connections between the moisture contents of other serotypes or strains and their thermal resistance. Validation of the quantitative relations between moisture content of bacterial cells and their thermal resistance may help further investigation into the fundamental roles that water molecules play in denaturation of key protein components leading to thermal inactivation of bacterial pathogens.

Materials and methods

Preparation of freeze-dried of *S.* Enteritidis PT 30

344 The stock culture of *Salmonella* Enteritidis PT 30 (ATCC-1045) was acquired from Linda
345 Harris at the University of California, Davis. It was stored at -80 ℃ in tryptic soy broth
346 supplemented with 0.6% (w/v) yeast extract (TSBYE, Difco™, Detroit, MI, USA) and 20% (v/v)
347 glycerol.

348 The procedure to produce the biomass of freeze-dried of *S.* Enteritidis PT 30 was modified
349 based on a previous study (22). Briefly, a loop of thawed stock was inoculated into 9 mL TSBYE
350 with two consecutive transfers and incubated at 37 ℃ for 24 h. Three hundred µL of this aliquot
351 was transferred into a centrifuge tube with 30 mL TSBYE and incubated at 37 ℃ for 24 h. Then,
352 4 mL of the previous culture broth was further transferred into a sterile conical flask with 400
353 mL TSBYE and incubated at 37 ℃ for 24 h with constant shaking at 230 rpm. The enlarged
354 bacterial culture was further washed twice with sterile double-distilled water (ddH_2O) by
355 centrifuging at 6,000 ×g for 10 min at 4 ℃. The supernatant was discarded and the washed
356 pellets from a total of 1.2 L bacteria broth (3 conical flasks) were pooled and re-suspended in 6
357 mL sterile ddH_2O. Washing caused the reduction in population was ~0.06 $\log_{10}$ CFU/mL, and the
358 population in the concentrated bacterial suspension was ~12.3 $\log_{10}$ CFU/mL. One milliliter of
359 the above bacterial suspension (~12 $\log_{10}$ CFU/mL) was distributed into each sterile clear serum
360 vials (5 mL, 22 mm outer diameter, DWK Life Sciences Wheaton™, Millville, NJ, USA) and
361 then loosely sealed with 2-leg lyophilization stopper (DWK Life Sciences Wheaton™, Millville,
362 NJ, USA). Vials were pre-frozen in liquid nitrogen for a few minutes and then lyophilized at –
363 90 ℃ and 0.006 mBar for 48 h in a freeze-dryer (FreeZone Plus 4.5 Liter cascade benchtop
364 freeze dry system, Labconco Corporation, Kansas City, MO, USA).

365 Generally, about 2 grams of freeze-dried of *S.* Enteritidis PT 30 was harvested from the
366 vials after each batch of freeze-drying. All solids from each batch were collected into a 4 oz

367 sterilized Whirl-Pak bag (Nasco, Modesto, CA, USA), hand-mixed for 5 min to eliminate clumps
368 and further homogenized using a stomacher at 230 rpm for 3 min (Stomacher®, 400 Circulator,
369 Seward Laboratory Systems Inc., Norfolk, UK). Then, this homogeneous freeze-dried of *S.*
370 Enteritidis PT 30 powder (10~11 $\log_{10}$ CFU/g) was kept at – 80 ℃ until use (within one week).
371 Each batch of the sample was used in an individual thermal treatment. All repeated batches were
372 derived from the same cold stock and cultivated independently.

373 **Viability test of freeze-dried of *S.* Enteritidis PT 30**

374 The viability and population were determined immediately by placing 0.100 ± 0.010 g of
375 freeze-dried of *S.* Enteritidis PT 30 into a 2 mL sterile snaplock microtube (Axygen™, Union
376 City, CA, USA), and then mixing with 0.9 mL buffered peptone water (BPW, Difco™, Detroit,
377 MI, USA) to obtain a 10-fold dilution. The freeze-dried of *S.* Enteritidis PT 30 was fully mixed
378 with BPW by a subsequent vortex (Pulsing Vortex Mixer, Fisherbrand™, Waltham, MA, USA).
379 Serial dilutions were performed to proper levels, and then spread in duplicate for enumeration at
380 37 ℃ for 48 h on modified TSAYE plates (49), which was made up of tryptic soy agar (Difco™,
381 Detroit, MI, USA), 0.6% (w/v) yeast extract (Difco™, Detroit, MI, USA), 0.05% (w/v) ferric
382 ammonium citrate (Sigma-Aldrich, St. Louis, MO, USA) and 0.03% (w/v) sodium thiosulfate
383 (Sigma-Aldrich, St. Louis, MO, USA. Typical *Salmonella* cells exhibited colonies with dark
384 solid circles. The average number of viable colonies was expressed as CFU/g based on two
385 technical replicates.

386 **Conditioning freeze-dried of *S.* Enteritidis PT 30 biomass to different a_w**

387 The initial a_w of the above homogeneous freeze-dried of *S.* Enteritidis PT 30 powder was
388 less than 0.025 at room temperature (~21 ℃), as measured with a water activity meter (Aqualab,

389 Meter Group, Inc., Pullman, WA, USA). The X_W of freeze-dried of *S.* Enteritidis PT 30 was
390 determined by the oven drying method according to AOAC 925.10 (50).

391 In order to obtain freeze-dried of *S.* Enteritidis PT 30 powder samples with different a_w or
392 X_W levels, freeze-dried of *S.* Enteritidis PT 30 was evenly spread in each sample cup (~300 mg
393 for high-temperature-cells) or petri dish (~2 g for thermal treatments) and conditioned at room
394 temperature for 2-3 days in air-tight jars with saturated salt solution under various relative
395 humidity levels at room temperature (19). The saturated salt solutions of LiCl, CH_3COOK,
396 $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, $NaNO_2$, NaCl and KCl could respectively generate a consistent
397 relative humidity of 11.3%, 22.5%, 32.8%, 43.2%, 52.9%, 65.8%, 75.3% and 84.3% at room
398 temperature, which corresponded to the equivalent target a_w values of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6,
399 0.7 and 0.8 at room temperature, respectively. In measuring the moisture sorption isotherms by
400 high-temperature cells, freeze-dried of *S.* Enteritidis PT 30 were conditioned at all the above
401 relative humidity levels. For the thermal treatments, freeze-dried of *S.* Enteritidis PT 30 were
402 conditioned at four selected relative humidity levels (22.5%, 32.8%, 43.2%, and 65.8%). The
403 equilibration of the freeze-dried of *S.* Enteritidis PT 30 samples was verified by measuring the a_w
404 value using the water activity meter. Samples were used for all experiments after reaching the
405 target $a_w \pm 0.02$.

406 **Thermal treatments of freeze-dried of *S.* Enteritidis PT 30**

407 Thermal treatments of freeze-dried of *S.* Enteritidis PT 30 were performed at 80 °C using
408 the improved aluminum Thermal-Death-Time test cells (TDT II) in triplicate. The TDT II cells
409 have a thinner thickness (1.39 mm in height) and a larger cavity (31.18 mm in diameter) as
410 compared to the traditional TDT cells (51). During the heating process, the X_W of freeze-dried of

411 *S.* Enteritidis PT 30 should remain constant in well-sealed TDT II cells, while the a_w of freeze-
412 dried of *S.* Enteritidis PT 30 could change with increasing temperature.

413 Come-up time (CUT, min) is the time required for samples to reach the target temperature
414 within 0.5 ℃. It was measured using a specially designed TDT II cell fabricated with a 0.5 mm-
415 diameter thermocouple (Type T, OMEGA Engineering, Inc., Stamford, CT, USA) secured on the
416 top center of the lid. One hundred milligrams of freeze-dried of *S.* Enteritidis PT 30 was filled
417 into the cell and sealed. The core temperature of the freeze-dried of *S.* Enteritidis PT 30 in the
418 test cell was measured by a thermometer (Digiense DuuaLogR 99100-50, Cole-Parmer
419 Instruments Co., Vernon Hills, IL, USA) when subjecting to the thermal treatment at 80 ℃. The
420 determination of the CUT was carried out in triplicate.

421 Freeze-dried of *S.* Enteritidis PT 30 samples were conditioned for 2-3 days to reach the
422 target a_w (0.2, 0.3, 0.4 and 0.6). 0.100 ± 0.010 g of the equilibrated freeze-dried of *S.* Enteritidis
423 PT 30 was loaded into a TDT II cell and sealed. Thermal treatment at 80 ℃ was carried out by
424 immersing test cells in the pre-heated ethylene glycol bath circulator (Isotemp™ 5150 H24,
425 Fisherbrand™, PA, USA). At each a_w, duplicate TDT II cells were removed from the circulator
426 at 5 pre-determined sampling points (come-up time was regarded as 0 min) and immediately
427 cooled down in an ice water bath for 1 min. Thermal inactivation treatments were independently
428 repeated in triplicate.

429 Thermally treated freeze-dried of *S.* Enteritidis PT 30 was transferred from the test cell to a
430 2-mL sterile snaplock microtube (MCT-200-C, Axygen, Union City, CA, USA) and 0.9 mL of
431 sterile BPW was added to achieve a 10-fold dilution. Next, the bacterial suspension was
432 homogenized using a vortex and further serially diluted by 10-fold. The appropriate dilutions
433 were spread on modified TSAYE plates in two technical replicates and incubated at 37 ℃ for 48

434 h. The average number of viable colonies at each time point was converted to CFU/g based on
435 two technical replicates.

436 **Statistical analysis**

437 The first-order kinetic model (log-linear model, Eq. 1) and Weibull model (Eq. 2) were
438 applied to analyze the thermal inactivation (52).

439 $$log\left(\frac{N}{N_0}\right) = -\frac{t}{D} \quad (1)$$

440 $$log\left(\frac{N}{N_0}\right) = -(\frac{t}{\alpha})^{\beta} \quad (2)$$

441 where t is the thermal treatment time (min), N is the bacterial population (CFU/g) at time t, N_0
442 is the initial bacterial population at the come-up time (CFU/g), D is the time (min) required to
443 inactivate the microbial population by 90% at a given temperature, α is the scale parameter (min)
444 and β is the shape parameter.

445 The thermal-decimal-time (D-value) in min was estimated by above models and the
446 goodness of fit was evaluated by R^2 coefficient. Statistical analysis of standard deviation was
447 performed using Microsoft Excel (16.35, Microsoft)

448 **Determination of a_w changes of freeze-dried of *S.* Enteritidis PT 30 with increasing**
449 **temperatures**

450 The a_w changes of freeze-dried of *S.* Enteritidis PT 30 at different temperatures were
451 determined using aluminum, high-temperature cells (Meter Group, Inc., Pullman, WA, USA)
452 designed by Tadapaneni et al., (19). The a_w was measured by a capacitance-based relative
453 humidity and temperature sensor (Honeywell HumidIcon™, Morristown, NJ, USA) located on
454 the center of the inner side lid. The sample cup with pre-equilibrated freeze-dried of *S.*

455 Enteritidis PT 30 (300 mg) was placed in a high-temperature cell and sealed tightly to prevent
456 any leakage. High-temperature cells were kept at room temperature (~21 ℃), and then heated in
457 20 ℃ increments from 40 ℃ to 80 ℃ by immersing into a heated glycol bath (Isotemp™ 5150
458 H24, Fisherbrand™, PA, USA). The relative humidity and temperature of the headspace were
459 read every minute, and the equilibrium state at the respective temperature was achieved when
460 constant relative humidity values were obtained for at least 10 records. Then, the temperature
461 and relative humidity values were recorded, and the relative humidity value was considered to be
462 the corresponding a_w value of the equilibrated freeze-dried of *S.* Enteritidis PT 30 in this closed
463 system at a certain temperature (19). After finishing the above series of measurements, the X_W of
464 freeze-dried of *S.* Enteritidis PT 30 was determined after cooling the high-temperature cells
465 down to room temperature. All experiments were performed in triplicate.

466 **Clausius-Clapeyron equation and moisture sorption isotherms**

467 The experimental a_w data was fitted by a modified Clausius-Clapeyron equation (CCE)
468 according to Tadapaneni et al.(19):

469 $$a_{w2} = a_{w1} exp\left(\frac{q_{st}}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)\right) \quad (3)$$

470 where a_{w1} and a_{w2} are the water activity values of a sample with the same X_w at temperature T_1
471 and T_2 (K), respectively; R is the universal gas constant (8.314 J mol^{-1} K^{-1}); q_{st} is the net
472 isosteric heat of sorption (J/mol), which can be determined from the slope of plotted data (ln a_w
473 versus 1/T).

474 Data measured by high-temperature cell was further fitted according to the Guggenheim-
475 Anderson-de Boer (GAB) model (Eq. 4) to generate the moisture sorption isotherms, providing
476 the relationship between a_w and X_W (27).

$$\frac{X_w}{X_m} = \frac{CKa_w}{(1-Ka_w)(1-Ka_w+CKa_w)} \quad (4)$$

where X_m is the monolayer moisture content (g water/100 g dry solids) in bacterial cells; a_w is the corresponding water activity value at X_w; C is a heat constant, ranging from 1 to 20; and K is a multilayer factor, ranging from 0.70 to 1.

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616 Table 1

617 The $D_{80°C}$ values of freeze-dried *S.* Enteritidis PT 30 at different moisture content.

$a_{w,20°C}$ ±0.02	Measured X_W (g water/100 g dry solids)	$a_{w,80°C}$ ± 0.02	*Linear model*		*Weibull model*		
			$D_{80°C}$ (min)	R^2	α (min)	β	R^2
0.20	7.7 ± 0.5	0.36	27.7 ± 0.7	0.98	13.8 ± 0.0	0.73 ± 0.11	0.98
0.30	9.2 ± 0.0	0.49	11.9 ± 0.2	0.97	6.1 ± 2.0	0.69 ± 0.10	0.96
0.40	12.4 ± 0.3	0.60	6.5 ± 1.1	0.92	1.9 ± 1.2	0.57 ± 0.06	0.85
0.60	15.7 ± 1.2	0.78	1.9 ± 0.6	0.84	0.4 ± 0.4	0.48 ±0.07	0.76

618 Note: Values are means ± standard deviations. The values of $a_{w,80°C}$ were predicted using CCE (Eq. 3). R^2: coefficient of
619 determination, higher values indicate a better fitness of model.
620

621 Table 2

622 The experimental values for the parameters in GAB model (Eq. 4) at different treatment

623 temperatures.

Treatment temperatures (°C)	GAB model parameters		
	X_m (g water/100 g dry solids)	C	K
20	10.0	9.42	0.70
40	9.1	8.43	0.71
60	7.6	9.00	0.75
80	7.5	6.67	0.73

624

Table 3

The estimated moisture contents of *S.* Enteritidis PT 30 using GAB model at 80 ℃ with reported *D*-values and $a_{w,80℃}$ in different low-a_w systems.

Low-a_w systems	$a_{w,80℃}$	$D_{80℃}$ values (min)	estimated X_W of *S.* Enteritidis PT 30 (g water/100 g dry solids)
Wheat Flour	0.47	12.2 ± 0.7	8.8
	0.68	4.9 ± 0.5	12.8
	0.78	1.2 ± 0.2	15.5
Almond Flour	0.43	27.3 ± 0.3	8.2
	0.54	21.2 ± 0.9	10.0
	0.63	11.1 ± 0.8	11.7
	0.81	0.8 ± 0.1	16.5
Whey Protein	0.41	17.5 ± 1.3	7.9
	0.62	10.6 ± 0.2	11.5
	0.74	5.1 ± 0.4	14.4
	0.87	1.5 ± 0.1	18.7
Honey Powder	0.18	35.6 ± 2.3	4.3
	0.31	27.3 ± 3.2	6.4
	0.40	19.6 ± 1.8	7.7
	0.50	14.4 ± 1.1	9.3
Silicon Dioxide	0.18	159.3 ± 5.8	4.1
	0.27	64.0 ± 0.2	5.7
	0.37	30.7 ± 1.0	7.3
	0.47	21.3 ± 1.4	8.8
	0.55	10.4 ± 0.3	10.1
	0.63	6.8 ± 0.3	11.8
	0.72	1.8 ± 0.1	13.8

Note: Means ± SD. The reported $a_{w,80℃}$ values and $D_{80℃}$ values for these low-a_w systems were derived from Alshammari et al., Liu et al. and Xu et al. (11, 14, 23).

631 Table 4

632 Comparison of thermal-resistance-related parameters in freeze-dried *S.* Enteritidis PT 30 and

633 low-a_w systems.

$a_{w,20℃}$	Low-a_w systems	$a_{w,80℃}$[1]	*estimated* X_W of *S.* PT 30[2] (g water/100 g dry solids)	$D_{80\,℃}$ values[3] (min)
0.45	Freeze-dried *Salmonella*	0.65	12.2	5.9 ± 0.8
	Wheat Flour	0.68	12.8	4.9 ± 0.5
	Almond Flour	0.54	10.0	21.2 ± 0.9
0.60	Freeze-dried *Salmonella*	0.78	15.5	2.1 ± 0.4
	Wheat Flour	0.78	15.5	1.2 ± 0.2
	Almond Flour	0.63	11.7	11.1 ± 0.8

634 [1]: the $a_{w,80℃}$ values of freeze-dried *S.* Enteritidis PT 30 were predicted using CCE (Eq. 3) and the
635 that for wheat flour and almond flour were derived from Xu et al. (11).
636 [2]: the X_W of *S.* Enteritidis PT 30 were obtained from GAB model (Eq. 4) at 80 ℃.
637 [3]: the $D_{80\,℃}$ values of freeze-dried *S.* were estimated from experiments-based regression as
638 shown in Fig. 3, and the $D_{80℃}$ values for wheat flour and almond flour were derived from Xu et
639 al. (11).
640

641 **Figure Legends**

642 **Fig. 2.** Illustration of moisture exchange between *Salmonella* cells and different
643 microenvironments during heating (created with BioRender.com).

644 **Fig. 2.** The representative thermal inactivation curves of freeze-dried *S.* Enteritidis PT 30 at 80 °C
645 in improved thermal-death-time test cells (TDT II) (X_W, moisture content, g water/100 g
646 dry solids; $a_{w,20°C}$, equivalent target water activity at room temperature after relative-
647 humidity-controlled equilibration).

648 **Fig. 3.** The $D_{80°C}$ values of freeze-dried *S.* Enteritidis PT 30 at different moisture contents (X_W, g
649 water/100 g dry solids). Exponential trend was observed in $D_{80°C}$-values as function of X_W
650 (○).

651 **Fig. 4.** Temperature-dependent changes in water activity of freeze-dried *S.* Enteritidis PT 30 at
652 different moisture contents (X_W, g water/100 g dry solids) (n=3). Open symbols represent
653 the average aw values as measured by high-temperature-cell (HTC), and dashed lines are
654 derived from Eq. (3).

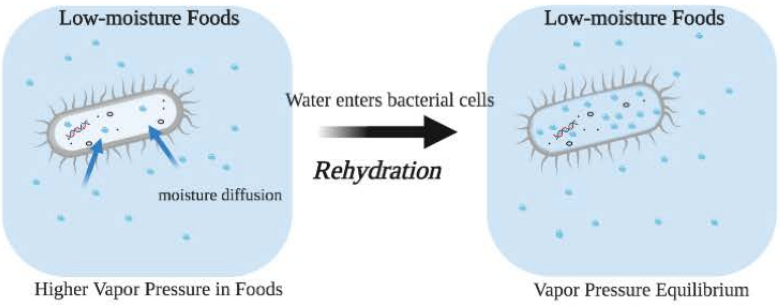
655 **Fig. 5.** Moisture adsorption isotherms of freeze-dried *S.* Enteritidis PT 30 at different
656 temperatures. The scattered data were generated using high-temperature-cells and the
657 dashed lines represent the curves fitted by the GAB model (Eq. 4).

658 **Fig. 6.** Net isosteric heat of sorption for freeze-dried *S.* Enteritidis PT 30 and several
659 representative food systems. The hollow circles (○) were the calculated data based on
660 measurements for freeze-dried *S.* Enteritidis PT 30. Both solid curve and dashed curves
661 were generated by the fitting equations of q_{st}. Fitting equations of food systems were
662 credited by Jin et al. and Tadapaneni et al. (18, 19).

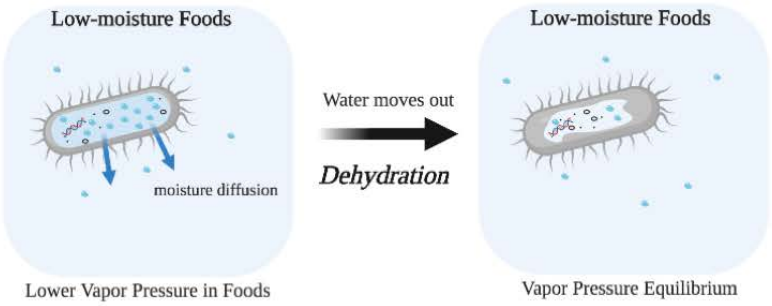
663 **Fig. 7.** The comparison of logarithmic $D_{80℃}$-values of *S.* Enteritidis PT 30 against the moisture
664 content in this study and other reported low-a_w systems. The gray shadow area represents
665 the confidence interval (95%) of Log $D_{80℃}$-values for freeze-dried *S.* Enteritidis PT 30.
666 The dark solid line represents the linear regression of logarithmic $D_{80℃}$-values for freeze-
667 dried *S.* Enteritidis PT 30 against its measured moisture content, the red dashed line
668 represents that with regard to the estimated moisture content of *S.* Enteritidis PT 30 for
669 other reported low-a_w systems.

670

Applied and Environmental Microbiology



(A) Higher a_w in low-moisture foods than bacterial cells at thermal-treatment temperature



(B) Lower a_w in low-moisture foods than bacterial cells at thermal-treatment temperature